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STUDIES OF A PURINE- OR HISTIDINE-REQUIRING MUTANT OF ESCHERICHIA COLI*

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In their work on histidine and purine requirements of *Streptococcus faecalis*, *Lactobacillus arabinosus*, and *Lactobacillus casei*, Broquist and Snell (1) have shown that, under certain conditions, purines could replace histidine, but histidine could not substitute for purines. The interrelation between purine and histidine metabolism has now been studied with a strain of *Escherichia coli* (W74) having a nutritional requirement which can be satisfied by either histidine or purines. The mutant used in these experiments was *E. coli* W74, isolated by Witkin and Kennedy (2). The mutant was characterized at isolation as a histidine-requiring auxotroph. Further study in this laboratory showed that purines could replace histidine in supporting growth.

EXPERIMENTAL

The bacteria were grown on the liquid minimal medium recommended by Gray and Tatum (3), to which the supplement under study was added. The cultures were grown in 25 ml. Erlenmeyer flasks provided with standardized side arms for turbidimetric readings. These flasks were agitated in a Warburg bath at 37°.

The flasks were inoculated (unless otherwise stated) with organisms grown with optimal concentration of histidine and washed twice with saline. The whole series of growth experiments reported here was obtained from cultures which had undergone two successive transfers made during the logarithmic phase.

The inoculum was calculated so as to give a final concentration of about 10^7 organisms per ml.; the turbidity of such a suspension is just detectable. The turbidity of the growing culture was recorded every half hour. The $\log_2$ of the turbidity was used to plot growth *versus* time curves (Fig. 1), from which the generation time was calculated (4). We have used this

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growth constant for comparative purposes rather than the total amount of growth, since generation time is more sensitive to concentration change and to the nature of the supplement than is the total amount of growth. Minimal generation time varies greatly with different supplements, while maximal total amount of growth is approximately the same with all.

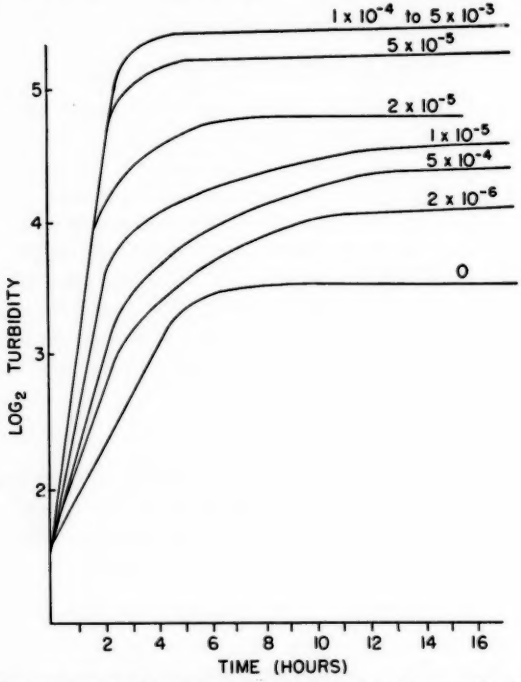

FIG. 1. Effect of varying histidine concentrations on the growth of *E. coli* W74

The following abbreviations have been used: DAP for diaminopurine, AMP-2′, 3′, or 5′ for adenosine-2′-, 3′-, or 5′-phosphate, respectively, and GMP-3′ for guanosine-3′-phosphate.

Results

E. coli W74 does not seem to have a complete biosynthetic block, for it can grow to a small extent on unsupplemented minimal media. The generation time varies from 3 to 5 hours, although the total amount of growth is more reproducible. The same results can be obtained regardless of whether the inoculum was previously grown on optimal concentra-

tions of histidine or was transferred several times in unsupplemented minimal medium: this indicates that the growth obtained is not due to carrying over histidine.

Very low concentrations of histidine promote a consistent decrease of the generation time and an increase of the total amount of growth, with the optimum at about 1×10^{-4} M (Fig. 1). The generation time at this and the above concentrations varies in different experiments from 42 to 60

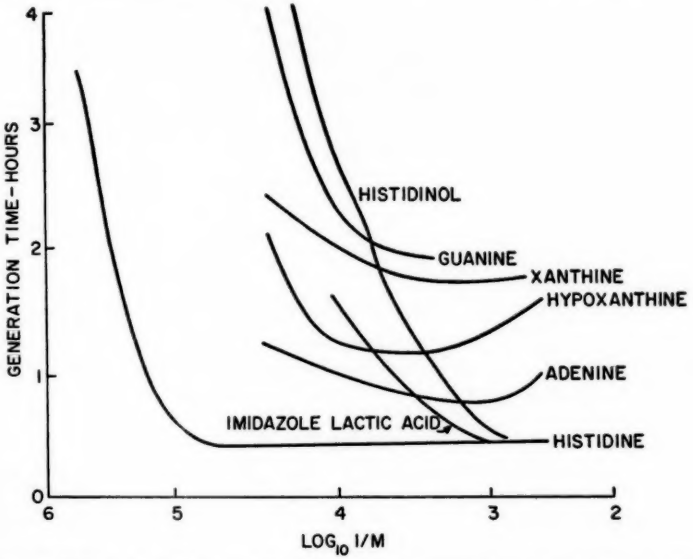

FIG. 2. Effect of purines and histidine on the generation time of *E. coli* W74

minutes. Under the same experimental conditions, the generation time of the wild strain varies from 42 to 54 minutes.

We have compared the efficiency of histidine and purines in promoting growth of strain W74 by comparing the effects of varying concentrations on generation time (Fig. 2) and total amount of growth (Fig. 3). Histidine provides by far the fastest growth at any given concentration; adenine and hypoxanthine are next, but these latter compounds fail to reduce the generation time to that obtained with histidine, while xanthine and guanine provide a much slower growth. If we compare the total amount of growth obtained with these compounds, however, the results are not as clear-cut as the data for generation time.

The effects on the growth of strain W74 of ribosides and ribotides have

also been compared (Table I). Adenosine-3′-phosphate provides more rapid growth than adenine or adenosine, while the 2′ isomer does not give better growth than the unsupplemented medium does. Furthermore, it

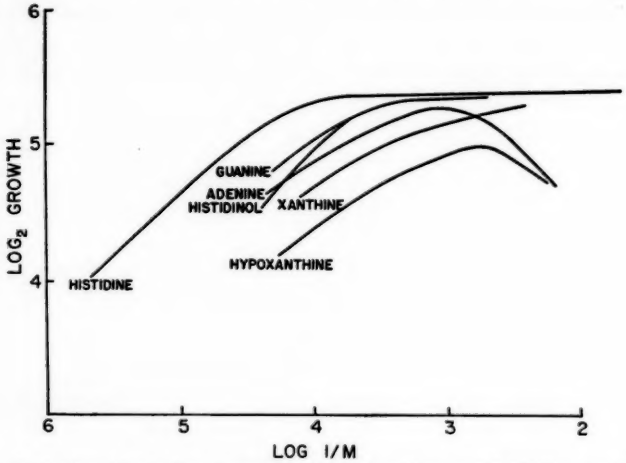

FIG. 3. Action of varying concentrations of purines and histidine on the total amount of growth of *E. coli* W74.

TABLE I

Response of E. Coli W74 to Various Adenine Derivatives

Supplement, 4×10^{-4} M	Generation time	Total amount of growth, $\log_2$ turbidity
	min.	
Histidine	60	5.4
AMP-3′	78	5.1
Adenine	90	5.1
Adenosine	90	4.9
ATP	114	4.5
AMP-5′	120	4.5
Coenzyme A	140	4.2
AMP-2′	180	3.5
Coenzyme I	180	3.5
Control	180	3.5

has been shown that the purine moieties of the 3′ isomers are extensively incorporated into the pentose nucleic acid of this organism (5). These results confirm the biological difference between the 2′- and 3′-nucleotide isomers already noticed in the case of *L. casei* (6) and *Bacillus subtilis* (7).

The 5′ isomer and adenosine triphosphate permit better growth than does the unsupplemented medium, but they are not as effective as adenylic acid-3′ or adenosine.

The curves for generation time per concentration and the total amount of growth per concentration with adenine and hypoxanthine show a peculiar feature: at concentrations greater than 10^{-3} M, the former increases again and the latter decreases (Figs. 2 and 3). This toxic effect has not

TABLE II

Inhibitory Effect of Adenine on Histidine-Supported Growth of E. coli W74

Adenine	Histidine	Total amount of growth, adenine alone, turbidity units	Generation time		
			Histidine alone	Mixture	Histidine + adenine / Histidine alone
M	M		min.	min.	
4×10^{-5}	4×10^{-5}	90	54	60	1.1
4×10^{-5}	4×10^{-4}	90	48	48	1
4×10^{-5}	4×10^{-3}	90	48	48	1
5×10^{-5}	2×10^{-5}	90	48	48	1
5×10^{-5}	5×10^{-5}	90	42	42	1
1×10^{-4}	2×10^{-5}	90	48	48	1
1×10^{-4}	5×10^{-5}	90	42	42	1
2×10^{-4}	2×10^{-5}	78	48	42	1
2×10^{-4}	5×10^{-5}	78	42	42	1
4×10^{-4}	4×10^{-5}	66	48	48	1
4×10^{-4}	4×10^{-4}	66	48	48	1
4×10^{-4}	4×10^{-3}	66	48	48	1
3×10^{-3}	2×10^{-5}	96	50	108	2.1
3×10^{-3}	5×10^{-5}	96	48	88	1.8
3×10^{-3}	1×10^{-4}	96	48	70	1.45
3×10^{-3}	1×10^{-3}	96	48	60	1.25
4×10^{-3}	4×10^{-5}	96	48	78	1.6
4×10^{-3}	4×10^{-4}	96	48	60	1.25

been found with histidine or xanthine (the insolubility of guanine prevented its being tested at those concentrations). The toxic action of high concentrations of adenine and hypoxanthine is even more evident when the bacteria are grown on a mixture of histidine and these purines. At low purine concentration, the generation time is that obtained with histidine alone, while for concentrations above 10^{-3} M it increases (Table II). Adenine has no inhibitory action on the wild strain of *E. coli*, but has been found to inhibit growth of guanineless mutants of *Ophiostoma* (8), *B. subtilis* (7), *S. faecalis* (9), and *Aerobacter aerogenes*.[1] In all the other instances, when the bacteria were grown on a mixture of purines or their

[1] B. Magasanik, private communication.

ribosides and histidine, the generation time found was one which corresponded to the concentration of histidine used. It was never shorter, as

TABLE III

Effect of Adenine Derivatives on Histidine-Supported Growth

Histidine	Supplement	Adenine-5′-PO_4	Adenosine triphosphate	Coenzyme A	Adenine-2′-PO_4	Adenine-3′-PO_4	Coenzyme I
M	M						
4×10^{-4}	4×10^{-4}	0.7			0.7	1	
4×10^{-4}	4×10^{-4}	0.6			0.95	1	
1.5×10^{-4}	1.5×10^{-4}	0.95	0.78	0.9	0.9	0.95	
2×10^{-4}	2×10^{-4}	0.94	0.87	0.94	1	1	1
2×10^{-4}	2×10^{-4}	0.84	0.95	0.95	1.05		
2×10^{-4}	2×10^{-5}				1	1	
2×10^{-4}	2×10^{-4}				1	1	
2×10^{-4}	4×10^{-4}				1		
2×10^{-4}	2×10^{-5}		0.87	0.87	1		
2×10^{-4}	2×10^{-4}		0.75	0.87	1		

The results are expressed as (generation time in a medium containing histidine + supplement)/(generation time in a medium with histidine alone).

TABLE IV

Growth of E. coli on Various Nitrogen Sources

Supplement, 1×10^{-3} M	Increase in No. of bacteria in 24 hrs., turbidity units	
	W74	Wild strain
Adenine	18	22
Xanthine	10	7
Hypoxanthine	3	0
Guanine	2	1
Adenosine	6	7.5
AMP-3′	6	7
AMP-2′	0	1
Inosine	0	
DAP riboside	0	2
Histidine	0	2
Histidinol	0	
Histamine	0	0
4-Amino-5-imidazolecarboxamide	0	
4-Amino-5-imidazolecarboxamidine	0	

would be expected if both substrates were used at the same time without synergism. But, when *E. coli* W74 was grown on a mixture of histidine

and purine ribotides, the generation time was shorter than with histidine alone (Table III). This was especially noticeable with AMP-5′, ATP, and coenzyme A; AMP-3′ did not modify the generation time, while results with AMP-2′ were erratic.

We have also tested the ability of several possible purine or histidine precursors, 4-amino-5-imidazolecarboxamide, 4-amino-5-imidazolecarboxamidine, 5-imidazolecarboxylic acid, 5-imidazolelactic acid, histamine, and histidinol, to support growth of strain W74. Of these, only histidinol and 5-imidazolelactic acid support better growth than the unsupplemented medium. As can be seen in Fig. 2, even if the concentration needed is 80- to 100-fold greater than in the case of histidine, these compounds are the only ones among those tested which will provide as short a minimal generation time as histidine does.

Histidine and purine ribosides and ribotides were also tested for their ability to serve as the sole nitrogen source for *E. coli* W74 (Table IV). Only adenine, AMP-3′, adenosine, and xanthine support growth, the total amount of growth increasing with the concentration of the supplement. The ability of strain W74 to degrade these substrates and utilize them as the sole nitrogen source is very similar to that of the wild strain (Table IV).

DISCUSSION

The growth pattern of *E. coli* W74 on histidine and purines, as shown by curves of the generation time *versus* concentration, suggests strongly that the lack of ability to synthesize histidine is the main biochemical deficiency of this mutant. No purine, ribotide, or riboside is as efficient as histidine in reducing the generation time; none of them is able to return the culture to "normality;" that is, to the generation time of the parent strain. This can only be achieved with histidine, a known histidine precursor, histidinol (10), or a known histidine substitute, 5-imidazolelactic acid (11, 12). Results of experiments in which the bacteria were grown on a mixture of histidine and purines or their ribotides seem to indicate that histidine is used in preference to the other supplement. Studies on the incorporation of labeled compounds into the nucleic acids of these bacteria (13) support this conclusion.

The ability to use purines as substitutes for histidine seems to be a unique peculiarity of this mutant: none of seven other histidineless mutants tested (three *E. coli* and four *B. subtilis*) would grow either on purines or on 5-imidazolelactic acid. The only other reported instance in which microorganisms could use purines as histidine substitutes is the work of Broquist and Snell (1). In this case also, the bacteria could use imidazolepyruvic and lactic acids (in the presence of pyridoxal) instead of histidine. There seems to be a definite relationship between the ability to use purines

and imidazolepyruvic or lactic acid as histidine substitutes. This suggests that perhaps purines and these last two imidazole derivatives might constitute a second and distinct pathway of histidine synthesis independent of histidinol.

The ability of *E. coli* W74 to grow on purines instead of histidine could also account for its slow growth on supplemented medium. As this bacterium can synthesize its own purines, it is possible that the synthesis does not require the presence of histidine (or histidine-containing enzyme) and that the purines thus synthesized are then converted to histidine, allowing bacterial growth.

SUMMARY

The growth responses and nutritional requirements of a mutant of *Escherichia coli* have been described. The organism's nutritional requirement can be met by histidine or one of several purines. The response to purine derivatives has been determined. The data indicate a purine-histidine interrelationship.

The authors wish to express their gratitude to Dr. C. Chester Stock, in whose department this work was carried out, and to Dr. George Bosworth Brown for much helpful advice and discussion.

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A PURINE-HISTIDINE RELATIONSHIP IN ESCHERICHIA COLI*

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The tracer technique of studying nucleic acid biosynthesis has yielded valuable information when applied to *Lactobacillus casei* (1–4). It was felt that additional information might be obtained by the use of a bacterium such as *Escherichia coli*, which requires a less complex growth medium. This paper presents the results of incorporation studies that have been carried out with *E. coli* B and a purineless mutant of this organism.

EXPERIMENTAL

The glycine-2-C^{14} was obtained from Tracerlab, Inc. Adenine-8-C^{14} was synthesized by cyclization of 5-formamido-4,6-diaminopyrimidine (5). The mutant of *E. coli* B, W74, was obtained from Dr. E. Witkin, and its nutritional requirements have been described (6).

The bacteria were grown in the glucose-salts medium of Gray and Tatum (7), modified by the omission of asparagine. The seed cultures were prepared by two serial transplants in the liquid medium used. Washed saline suspensions of the bacteria were then added to the experimental medium in Erlenmeyer flasks.

The bacteria were grown with continuous aeration for 16 hours at 37° and were then collected by centrifugation. The cells were washed with cold trichloroacetic acid, defatted with alcohol and ether, and hydrolyzed at 37° for 24 hours with 1 ml. of 1 N NaOH per 180 mg. of bacterial residue (8). The degraded pentose nucleic acid (PNA) was separated from the deoxypentose nucleic acid (DNA) by acidification of the solution with HCl, followed by the addition of 1.5 volumes of ethanol. The DNA was collected by centrifugation. The supernatant liquid containing the PNA fragments was made 1 N with respect to sulfuric acid and was heated at about 100° for 1 hour to hydrolyze the purine nucleotides. The DNA was washed with dilute acid and was similarly hydrolyzed with 1 N sulfuric acid.

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The hydrolysates of both the PNA and DNA were made ammoniacal, and the purines were precipitated as their silver salts. The free purines were regenerated with hydrochloric acid and separated by paper chromatography. The radioactivities were determined as described previously (2, 3); thin films on aluminum planchets were measured in an internal Geiger-Müller flow counter (Radiation Counter Laboratories, mark 12, model 1, helium-isobutane gas). The radioactivity on all planchets was sufficient to result in recorded activity of at least twice the background. The activities were determined to within standard errors of less than 5 per cent (9). Activities are reported as relative specific activities (R. S. A.).

$$\text{R. S. A.} = \frac{\text{c.p.m. per mole isolated compound}}{\text{c.p.m. per mole proffered compound}} \times 100$$

For the N^{15} determinations a Consolidated-Nier mass spectrometer, model 21-201, standard deviation 0.001 atom per cent, was employed. The following abbreviations have been used: AMP-5′ = adenosine-5′-phosphate; AMP-2′ = adenosine-2′-phosphate; AMP-3′ = adenosine-3′-phosphate; DAP = diaminopurine; ATP = adenosine triphosphate.

RESULTS AND DISCUSSION

Histidine-Purine Interrelationship—The ability of histidine to influence the synthesis of nucleic acid purines in the mutant *E. coli* W74 is shown in Table I. This organism is fully able to synthesize purines in a histidine-containing medium. The addition of adenine instead of histidine to the medium reduces the utilization of glycine-2-C^{14} for purine synthesis,[1] and the reduction in the case of the PNA adenine is the more striking (Experiments 1 and 2 *versus* Experiments 3 and 4). If the proffered quantity of histidine or adenine is increased (Experiments 1 and 3 *versus* Experiments 2 and 4) the *de novo* synthesis of PNA purines from glycine decreases. This is more noticeable in the case of adenine and is similar to the effect of exogenous bases in sparing *de novo* synthesis of purines by *L. casei* (2).

As shown in Table I, the *de novo* synthesis of guanine from glycine (in the presence of adenine) is strongly reduced by the substitution of histidine for half of the exogenous adenine (about 20 per cent in Experiment 4 *versus* Experiment 5). In Experiment 6, the relative specific activity of the gua-

[1] The utilization of glycine serves as an over-all measure of *de novo* synthesis. If no exogenous purine source is available, the label introduced from a given amount of glycine-2-C^{14} is equivalent to 100 per cent *de novo* synthesis (2, 10). Any reduction in this value must represent a corresponding increase in the use of some exogenous source. As a first approximation, the data in Experiments 1 and 2 can be taken as measures of total *de novo* synthesis. The per cent decrease in these values in other experiments, therefore, is equal to the per cent derived from the purine added to the medium.

nine was less than 100. These facts suggest either a sparing effect of histidine on the conversion of adenine to guanine or a direct conversion of histidine to the PNA purines, more particularly to guanine. The simultaneous addition of both histidine and adenine has almost completely eliminated synthesis *de novo* (Experiment 5).

In order further to elucidate which parts of the histidine moiety might be serving as a precursor of part of the nucleic acid purines, an experiment was performed in which the medium contained histidine at a concentration of

TABLE I

Adenine-Histidine Interrelationships in E. coli W74

Experiment No.	Compound added*	Amount in 300 ml. culture	Relative specific activity of PNA bases	
			Adenine	Guanine
		μmoles		
1	**Glycine**	80	89	78
	Histidine	45		
2	**Glycine**	80	80	76†
	Histidine	90		
3	**Glycine**	80	6	29
	Adenine	45		
4	**Glycine**	80	2	19
	Adenine	90		
5	**Glycine**	80	1	2†
	Adenine	45		
	Histidine	45		
6	**Adenine**	45	103	88†
	Histidine	45		

* The labeled compound is in bold-faced type.

† Average of two separate experiments which agreed within 10 per cent.

5×10^{-4} M and $N^{15}H_4^+$ at a concentration of 6×10^{-2} M (Table II). Previous work has shown that histidine cannot serve as a sole nitrogen source for this *E. coli* mutant (6). Therefore one would conclude that any nonspecific utilization of nitrogen would have to come from the ammonia-N^{15} in the medium, and reduction in N^{15} content of the isolated purines relative to that of the ammonia in the medium would have to result from specific utilization of 1 or more of the nitrogen atoms of the histidine molecule. The observed reductions in the possible N^{15} content (Table II) are evidence that histidine-N^{14} has served as a precursor for some part of the purine moiety.

It has been shown in other systems that there can be a partial utilization of carbon 2 of the histidine moiety for the synthesis of purine (11, 12).

The incorporation observed was of such a magnitude as to be theoretically explicable by assuming that carbon 2 is incorporated into a common 1-carbon pool. However, in the case of *E. coli* it is difficult to visualize any such simple explanation in view of the cumulative data in Table I and the N^{15} results in Table II, and it appears that a larger moiety of the histidine has been used for purine synthesis. Broquist and Snell (13) have presented presumptive evidence for the reverse transformation, *i.e.* that of purines to histidine, but there is as yet no way to ascertain the specific entities involved in that transformation either.

Ribotide Utilization—E. coli W74 can use AMP-3′ as a purine source for growth and is unable to use AMP-2′ (6). Furthermore it can, in com-

TABLE II

*Reduction in Utilization of $N^{15}H_4^+$ for Purine Synthesis by Exogenous Histidine by E. coli W74**

Isolated base	Relative isotope content†	Per cent derived from other N sources
PNA adenine	89	11
" guanine	89	11
DNA adenine	85	15
" guanine	82	18

* The nitrogen of the proffered ammonia contained 2.41 atom per cent excess of N^{15}. The ammonia concentration was 6×10^{-2} M; the histidine concentration was 5×10^{-4} M.

† $\frac{\text{Atom \% excess isolated compound}}{\text{Atom \% excess added ammonia}} \times 100.$

mon with the parent strain of *E. coli* B, incorporate the purine moiety of the 3′ isomer into its nucleic acid, but it cannot do so with AMP-2′ (10). The effect on the growth of *E. coli* W74 of AMP-5′, however, is quite different. This compound and closely related derivatives of it, *e.g.* coenzyme A and ATP, are able to shorten the generation time when added to a medium containing histidine. When these compounds were added to the medium containing glycine-2-C^{14}, their influence on nucleic acid synthesis was measured as is shown in Table III. This result is quite different from that found with AMP-2′ and AMP-3′ (10). The former compound did not serve as a purine source at all, and the latter was almost twice as effective as either of the 5′ derivatives (Table III). In studies with *L. casei* the adenine moiety neither of AMP-5′ nor its derivatives served as purine precursors (4). Using *Aerobacter aerogenes*, Brooke and Magasanik (14) have noted that under some conditions AMP-5′ is the purine ribotide best able to support the growth of a purineless mutant. The presence of

enzymes capable of degrading AMP-5′ in *E. coli* has been shown (15), as has an AMP-3′ phosphatase (16). The presence of such enzyme systems might explain the ready utilization of the purine moiety of the derivative, but does not suggest a mechanism whereby the generation time is reduced.

As a base-line against which to evaluate the data obtained with *E. coli* W74, we studied the ability of its parent strain, *E. coli* B, to use various purine precursors. Similar studies have been made by Koch *et al.* (17), but it was deemed advisable to carry out series of experiments under conditions directly comparable to those used with the mutant. The values

TABLE III

Effect of Adenosine-5′-phosphates on Purine Synthesis

Experiment No.	Compounds added*	Amount in 300 ml. culture	Per cent derived from adenylic acid†	
			Adenine	Guanine
		μmoles		
8	ATP	45		
	Histidine	45	60	37
	Glycine	80		
9	AMP-5′	45		
	Histidine	45	72	55
	Glycine	80		

* Labeled compound in bold-faced type.

† Defined as

$$100 = \left(\frac{\text{R. S. A. of isolated compound}}{\text{R. S. A. of isolated compound of control}} \times 100 \right)$$

where the control experiment was a duplicate save for the omission of the adenine nucleotide from the medium (see foot-note 1).

in Table IV were obtained by the technique of reciprocal labeling (2). The incorporations into PNA and DNA were essentially the same when both were measured and could be construed as indicative that the routes of interconversion of the purines of both PNA and DNA are for the most part identical (18).

In *E. coli* B, as is the case with *E. coli* W74, AMP-5′ served as a purine source and was much better as a source of adenine than it was of guanine. The utilizations of adenosine and inosine were very high and essentially the same for both precursors (Experiments 11 and 13). Both of these compounds served equally well as precursors of PNA adenine or guanine, and in the case of adenosine this extended to the DNA also. When guanosine was added to the growth medium, it too served as a purine source (Experiment 12). In this case, however, there was an imbalance, and a

much greater proportion of nucleic acid guanine was derived from the exogenous guanosine. It is interesting to note that here again the DNA and PNA adenine were similarly labeled; this same parallelism was observed with the DNA and PNA guanine. Diaminopurine riboside was (Experiment 14) utilized to a lesser extent than were any of the other ribosides studied, and it showed a great differential conversion to guanine.[2] The

TABLE IV

Synthesis of Nucleic Acid Purines by E. coli B

Experiment No.	Organism	Compounds added*	Amount in 300 ml. culture	Per cent derived from exogenous purine compound†			
				PNA		DNA	
				Adenine	Guanine	Adenine	Guanine
			μmoles				
10	B	AMP-5′	90	45	24	46	19
		Glycine	80				
11	"	Adenosine	90	89	85	89	86
		Glycine	80				
12	"	Guanosine	90	17	80	12	85
		Glycine	80				
13	"	Inosine	90	88	86		
		Glycine	80				
14	"	DAP riboside	90	3	30		
		Glycine	80				
15	W74	Histidine	45	90		90	
		Inosine	45				
		Glycine	80				
16	"	Histidine	45	27		19	
		DAP riboside	45				
		Glycine	80				

* Labeled compound in bold-faced type.

† See Table III. In Experiments 15 and 16 the values calculated are for the purine compound plus histidine.

incorporation of two of these ribosides (inosine and DAP riboside) into the nucleic acid adenine of strain W74 has also been determined (Experiments 15 and 16). Here again, the essential equality of PNA-DNA is maintained.[3]

[2] In Experiment 14 there was a slight inhibition of growth. Under the conditions of the experiment generation time increases from 42 to 54 minutes and total growth diminishes from 5.2 to 4.9.

[3] The difference between 27 and 19 per cent is not as significant as it might appear; the measured relative specific activities were 100 − 73 and 81.

The restricted transformation of the precursors into the two pentose nucleic acid purine bases in *E. coli* (both strains B and W74) is similar to that observed by Koch *et al.* (17), and the contrast with the very ready interconversion in *L. casei* should again be noted (1–4).

It is apparent that the mutant *E. coli* W74 has a metabolic block of such a nature as to demonstrate a definite purine-histidine relationship reminiscent of that observed by Broquist and Snell (13). It is difficult to assess its nature thoroughly. It may be fundamentally a precursor-product relationship magnified by the organism's inability to make some common intermediate. It is also possible that one of these compounds is part of some larger molecule, *e.g.* a coenzyme, which mediates the synthesis of the other.

SUMMARY

The abilities of *Escherichia coli* B to utilize various purine-containing compounds for the synthesis of nucleic acid purines have been studied. The results have been compared to those obtained with a mutant, *E. coli* W74, which requires either histidine or a purine for growth. The mutant and its parent have the same ability to interconvert exogenous purines. The ability of the mutant to utilize histidine carbon as a precursor of the nucleic acid purines has been detected. The sparing of $N^{15}H_4^+$ utilization by histidine indicates that this must involve the utilization of more than merely the amidine carbon atom of the histidine.

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SOME PROTEOLYTIC ACTIVITIES OF SNAKE VENOMS*

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Most snake venoms manifest proteolytic activity. Their relation to trypsin in clotting processes was intensively investigated by Eagle in 1937 (1). Continuing research of this nature has been, in general, relatively limited or supplementary to other problems concerned with venoms. Many of these studies have been reviewed by Jánszky (2). It was of interest to extend previous work by investigating the proteolytic activity of a series of venoms in terms of their action on various substrates and in the presence of several trypsin inhibitors. The latter enzyme was often utilized as a reference. It was found that the venom proteases have properties which in many respects simulate those of trypsin, but may be clearly differentiated by the action of inhibitors.

EXPERIMENTAL

Fifteen venoms encompassing those most common to Brazil and North America were utilized. These were available as either lyophilized or vacuum-dried specimens.[1] Solutions of the venoms were prepared immediately before use, since considerable loss of activity is often experienced on aging of solutions.

The sample of crystalline trypsin (Armour) utilized was dialyzed extensively against 0.001 N HCl and maintained at 1–2°. Crystalline chymotrypsin (Armour) was reconstituted to solutions of desired potency immediately before use. Soy bean trypsin inhibitor (SBI) crystallized five times, the sodium salt of ethylenediaminetetraacetic acid (EDTA or Versene), and ovomucoid were employed as protease inhibitors. Two fractions of the latter protein, of which a crude and a pure preparation showed equivalent antitryptic activities, were separated according to the method of Fredericq and Deutsch (3).

Proteolysis of urea-denatured hemoglobin was carried out by the method

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[1] A portion of the venoms was generously supplied by the Instituto Butantan and by Wyeth, Inc.

of Anson (4). The extent of digestion was evaluated colorimetrically by recourse to the usual Folin-Ciocalteu reagent or by determination of the absorption at 280 mμ of the trichloroacetic acid filtrate with the Beckman spectrophotometer.

The fibrinogen preparation employed was Armour's Fraction I of bovine plasma.[2] Solutions of it were allowed to react with bovine thrombin[2] to provide fibrin.

A globulin fraction of bovine serum served as the substrate for bradykinin formation. It represented the pseudoglobulin portion of the serum proteins insoluble in half saturated $(NH_4)_2SO_4$. This fraction was reconstituted to one-fourth its original serum volume and adjusted to pH 7.4. 4.5 ml. of such a preparation were incubated at 37° with 0.5 mg. of dry venom dissolved in 0.5 ml. of Tyrode's solution. From 0.02 to 0.1 ml. of this mixture was removed at intervals and assayed for the hypotensive and smooth muscle-stimulating principle designated as bradykinin (5, 6). The assay method employed a section of guinea pig ileum, previously desensitized to the particular venom employed, as the contractile element (5, 7). A bradykinin preparation containing 5.2 units per mg. (6) was used as both standard and substrate for evaluating formation from the globulin fraction and the rate of destruction respectively. The latter was carried out essentially by the method described by Hamberg and Rocha e Silva (7).

Benzoyl-L-arginine ethyl ester[3] and acetyl-L-tyrosine ethyl ester[3] were utilized as the synthetic substrates in measurements of tryptic and chymotryptic activity respectively. These were carried out by the potentiometric null point method of Schwert *et al.* (8). 3 ml. of 0.005 M substrate were employed in each assay, and the time required for half destruction of the substrate was arbitrarily taken as the end-point.

Results

Hemoglobin Digestion—A wide range of activities was experienced, as shown by the data of Table I. The most active venom, that of *Agkistrodon piscivorus piscivorus* (the cottonmouth moccasin), showed about 5 per cent of the activity of crystalline trypsin. Both the white and yellow forms of the venom of *Crotalus terrificus terrificus* had only a weak and barely perceptible proteolytic activity. In agreement with the work of Green and Neurath (9), EDTA did not inhibit the dialyzed trypsin. However, all of the venom proteases with the exception of the two species mentioned above were strongly inhibited by this substance. The effect of EDTA is dependent on concentration and on time of incubation. In Fig. 1 the

[2] These products were a gift of Armour and Company.

[3] The authors are indebted to Dr. Hans Neurath for these compounds.

effect of concentration of this substance on the proteolysis of hemoglobin by the venom of *A. piscivorus piscivorus* is shown. The EDTA was added

TABLE I

Proteolytic Activity of Venoms of Various Snakes

Venom*	Activity†						Clotting *vs.* Hb digestion
	Hb	Hb + EDTA‡	BAEE	Clotting§	Bradykinin		
					Formation	Destruction	
Trypsin	610	610 (0.02)	56	None	5.2	0.14	
Chymotrypsin					0	94.1	
A. piscivorus piscivorus	30	17 (0.25)	8.0	None	13.5	0.0	
L. muta	29	18 (0.75)	8.3	12	41.1	0.10	0.41
C. atrox	21	3.2 (0.3)	2.8	None	46.2	5.60	
" *horridus*	17	2.7 (0.4)	5.9	2	31.2	0.44	0.12
A. contortrix	13	9 (0.4)	11.6	None	53.0	0.0	
B. neuweidii	7.9	3 (1.0)	2.2	1.6	0.9	0.04	0.2
" *jararacussu*	4.4	0.8 (1.5)	3.8	3		0.15	0.68
" *jararaca*	3.1	0.8 (2.0)	3.7	11	17.2	0.72	3.5
" *insularis*	2.5	0.8 (4.0)	2.9	3.6	1.4	0.50	3.4
" *cotiara*	2.1	0.6 (1.5)	7.9	2.3	2.5	0.93	1.1
" *alternatus*	1.5	0.3 (12)	0.4	0.25	0.5	0.35	0.17
" *atrox*	1.5	0.7 (3.0)	3.8	7.8	3.7	0.49	5.2
C. adamanteus	1.0	0.3 (10)	2.3	7	36.3	0.07	7
" *terrificus terrificus* (yellow)	0.01	0.01 (20)	0.7	3	1.7	0.30	300
C. terrificus terrificus (white)	0.005	0.005 (20)	1.4	2	2.5	0.23	400

* *Agkistrodon* (*A.*), *Crotalus* (*C.*), *Bothrops* (*B.*), and *Lachesis* (*L.*).

† For hemoglobin, milliequivalents of tyrosine $\times 10^4$ per mg. per 15 minutes liberated at 37°, pH 7.4; for BAEE, moles hydrolyzed $\times 10^6$ per mg. per minute at 22°; for bradykinin, units formed or destroyed per mg. of venom per minute at 37°.

‡ Concentration of EDTA 5.6×10^{-4} M. The figures in parentheses indicate the mg. of venom used in each test.

§ The clotting activity is expressed as the reciprocal of the mg. of venom required to clot 1 ml. of a 300 mg. per cent solution of fibrinogen at pH 7.4 in 30 seconds at 25°.

to the substrate prior to the addition of venom. Incubation of venom with EDTA (3.4×10^{-3} M) for 15 minutes at 37° before addition of substrate completely inactivated the proteolytic activity. Addition of the chlorides of strontium, cobalt, barium, manganese, cadmium, magnesium, calcium, and zinc and of the nitrates of copper and cobalt in a final concentration of 1×10^{-2} M was ineffective in reversing EDTA action. Of

these, copper, manganese, cadmium, cobalt, and zinc inhibited the proteolysis by the venoms in varying degree. Hargreaves (10) has previously shown that Ca ions augment the clotting activity of *Bothrops jararaca* venom on plasma. More recently Rosenfeld and Jánszky (11) have found that EDTA inhibits the clotting action of venoms on fibrinogen and that this effect is reversed by Ca ion. In contrast to trypsin, the proteolytic activity of venoms on hemoglobin is not inhibited by ovomucoid or by SBI. In many experiments ovomucoid in amounts as low as 0.25 mg. per 6 ml. of reaction volume caused an enhancement of proteolysis by venoms ranging from 10 to 80 per cent. This effect was quite variable and attempts to regulate and study this stimulatory effect were unsuccess-

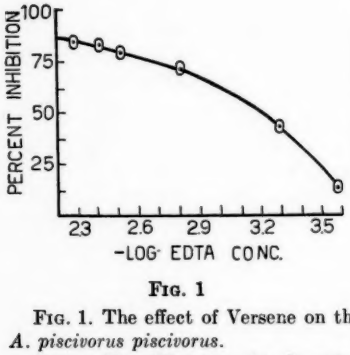

FIG. 1

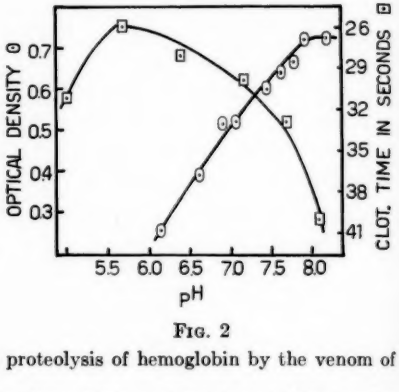

FIG. 2

FIG. 1. The effect of Versene on the proteolysis of hemoglobin by the venom of *A. piscivorus piscivorus*.

FIG. 2. The pH optima for digestion of hemoglobin and clotting of fibrinogen by the venom of *L. muta*.

ful. Taborda and Taborda (12) have previously reported that the addition of mucins to the venom of *B. jararaca* resulted in an enhancement of its proteolytic activity from 5 to 7 per cent.

Clotting of Fibrinogen—All of the venoms clotted fibrinogen with the exception of *A. piscivorus piscivorus*, *Agkistrodon contortrix*, *Crotalus horridus*, and *Crotalus atrox*. As previously reported by Eagle (1), these four venoms are typical of those acting like trypsin in that they convert fibrinogen into products not clottable by thrombin. It has been suggested that the proteolytic and fibrinogen-clotting activities of a given venom are related (2, 13). However, Taborda (14) has found that the proteolytic and clotting effects of a given venom have different susceptibilities to heat denaturation. The ratios of these two activities, as given in Table I, suggest that separate enzymes are concerned. The difference previously noted in the ability of calcium to reverse the effect of EDTA on the clotting

of fibrinogen but not on the proteolysis of hemoglobin is also suggestive that different enzymes may be involved. The pH optima of these two processes are quite different also, as shown by the data of Fig. 2 for the venom of *Lachesis muta*. This, however, is not strong evidence for separate enzymes. The pH optimum of thrombin varies considerably for the hydrolysis of two such relatively similar substrates as *p*-toluenesulfonylarginine methyl ester and lysine ethyl ester (15), and the pH optimum for trypsin varies for different proteins (16). Neither ovomucoid nor SBI inhibits the clotting of fibrinogen or of plasma by venoms. In contrast, both of these substances inhibit the ability of trypsin to clot plasma. We were unable to confirm the report of Croxatto (17) that SBI inhibits the clotting of plasma as effected by the venom of *Bothrops neuweidii*.

Digestion of Fibrinogen and Fibrin—The venoms which rapidly digest fibrinogen without the formation of a clot were noted to have a very weak action on fibrin. The action of several venoms and of trypsin on fibrinogen and on fibrin was studied quantitatively. Various amounts of venom were allowed to act on fibrinogen at pH 7.4 and 37°. At intervals samples were removed and pipetted into buffered thrombin solutions sufficiently acid to give a resultant pH near 5.8, at which point fibrinogenolysis did not take place with any appreciable rate, but thrombin was very active. The clot forming was removed quantitatively, dried, and weighed. Trypsin activity was stopped by the addition of ovomucoid.

Proteolysis of fibrin was determined by simultaneously adding a given amount of venom and approximately 15 units of thrombin per ml. of fibrinogen (300 mg. per cent) to a series of fibrinogen samples. Clotting took place within 5 seconds. The tubes were then incubated for 10 minutes at 37°, and at this time and at later intervals the proteolytic reaction was stopped by addition, with stirring, of sufficient acetate buffer of pH 4 to bring the pH to 5.8. The sample incubated for 10 minutes was designated the zero time sample, as under the above conditions all of the original fibrinogen has been converted to fibrin or material not capable of forming fibrin. Decreases in fibrin following this period were then indicative of fibrinolysis. The residual fibrin was removed at various intervals, dried, and weighed. In experiments utilizing trypsin the reaction was again stopped by rapidly stirring ovomucoid into the fibrin gel.

The data presented in Figs. 3, *A* and *B* indicate that the venoms and trypsin hydrolyze fibrinogen more rapidly than fibrin. A venom like that of *B. jararaca*, which clots fibrinogen, also has an action on this protein which converts it into non-clottable material. The addition of 2 mg. of this venom to 3 mg. of fibrinogen results in the formation of only a small amount of fibrin clot (Fig. 3, *A*). However, even in the presence of this large amount of venom, a small amount of fibrin persists after 3 hours of incubation.

An apparent complication of these experiments was the presence of small amounts of serum trypsin inhibitor in the fibrinogen preparation employed. Thus 10 γ of trypsin completely digest 3 mg. of fibrinogen to a non-clottable form in less than 5 minutes, whereas 4.3 γ destroy less than half of this amount of fibrinogen in 3 hours. The snake venom proteases are not affected by this substance. Eagle (1) has also shown that serum antithrombins do not inhibit the ability of certain venoms to clot fibrinogen or plasma.

Hydrolysis of Benzoyl-L-arginine Ethyl Ester (BAEE)—All of the venoms studied hydrolyzed this compound. Rocha e Silva and Andrade (18) have previously shown the tryptic-like action of the venoms of *C. terrificus terrificus*, *B. atrox*, and *B. jararaca* as related to the hydrolysis of benzoyl-L-argininamide. However, this compound and acetyl-L-leucinamide have

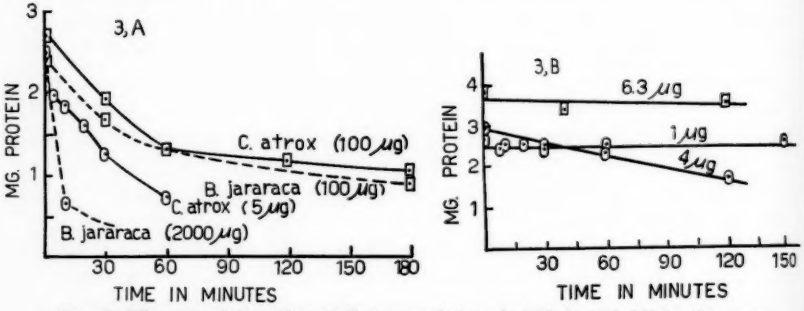

FIG. 3. The rate of digestion of fibrinogen (⊙) and of fibrin (⊡) (*A*) by the venoms of *C. atrox* and *B. jararaca* and (*B*) by trypsin.

been reported by Marsano, Croxatto, and Croxatto (19) to be resistant to the action of *B. neuweidii* venom.

As shown by Table I, the extent of activity of the venoms on BAEE is not always related to their action on hemoglobin. Thus *A. piscivorus piscivorus* venom was several times more active than that of *A. contortrix* on hemoglobin, but had far less activity on BAEE. Similar relations for other venoms may be seen in Table I. The action of both *C. terrificus terrificus* venoms on BAEE was likewise greater than was to be expected from their action on hemoglobin. Interestingly, EDTA had no effect over a wide range of concentrations on the splitting of BAEE. Ovomucoid and SBI, while inhibiting the effect of trypsin on this ester, likewise were ineffective on the venom proteases.

Hydrolysis of Acetyl-L-tyrosine Ethyl Ester—None of the venoms possessed significant chymotryptic activity. The most active venom, that of *A. contortrix*, showed less than 0.1 per cent of the activity of chymotrypsin.

Formation of Bradykinin—All of the venoms tested are able to liberate bradykinin. Some typical activities as exemplified by the release of this substance from the serum globulin substrate are illustrated by the data of Fig. 4. It can be seen that *C. atrox* and *C. terrificus terrificus* venoms also destroy the bradykinin formed, while *A. contortrix* is inactive in this respect. The action of these venoms on purified bradykinin is illustrated in Fig. 5.

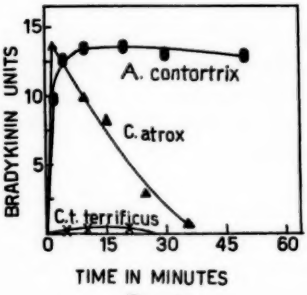

FIG. 4

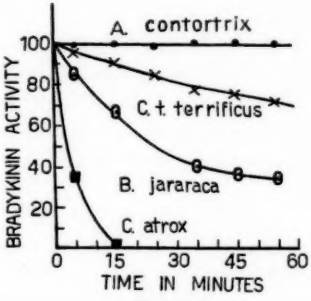

FIG. 5

FIG. 4. The liberation of bradykinin from a serum globulin fraction through the action of various venoms.

FIG. 5. The rate of destruction of bradykinin by various venoms.

TABLE II

Rate of Formation of Bradykinin from Serum Globulin Fraction by Action of Venom of C. atrox

Reaction time	Bradykinin activity	Total bradykinin liberated*
min.	*units per ml.*	*units per ml.*
3.5	14.3	16.2
9	10.6	15.5
15	8.6	16.9
25	2.8	16.6
30	1.4	18.2

* Corrected for destruction of bradykinin.

It is apparent that there are marked differences among the various venoms in their ability to destroy this substance. In order to compute the activity of a venom in forming bradykinin from the globulin substrate, a correction for the destruction by the venom and globulin fractions must be applied. The latter is very small. When these are applied to the data for *C. atrox* presented previously in Fig. 4, a rate of formation of bradykinin may be readily calculated, as shown by the results of Table II. The rate of formation and destruction of bradykinin by all of the venoms studied is also summarized in Table I. The different venoms are seen to exhibit

marked differences in activity. In general the venoms most active as regards the proteolysis of hemoglobin and the hydrolysis of BAEE have the highest levels of activity. However, as was true for the relation of hemoglobin and BAEE hydrolysis, many exceptions to this are apparent. One of the most notable is the activity of the venom of *Crotalus adamanteus*. The venom of this species possesses very weak activity for the splitting of hemoblogin and BAEE, but has a marked ability to form bradykinin (Table I).

The low activity of trypsin in the formation of bradykinin is probably due to the antitryptic action of the globulin fraction, as previously noted in the studies on digestion of fibrinogen. Neither ovomucoid nor SBI affects the action of the venoms in forming or destroying bradykinin, but both inhibit completely the action of trypsin.

DISCUSSION

The proteolytic activities of venoms have been shown to be more or less localized in specific electrophoretic components by Gonçalves and Polson (20) and by Gonçalves and Vieira (21). Further work of this nature has recently been reported by Grassmann and Hannig (22). The results of the present studies suggest that a given venom may contain several proteolytic enzymes. In particular it appears that the enzymes associated with the clotting of fibrinogen and the proteolysis of hemoglobin are separate entities. Attempts to separate the bradykinin-forming and destroying and the fibrinogenolytic and fibrinogen-clotting enzymes of several venoms into separate fractions by paper electrophoresis were unsuccessful.

It should be emphasized that the proteolytic activity by different collections of the venom of a given species of snake may present wide variations, and the results from various laboratories will thus be quite different. However, if a given venom protease functions to clot fibrinogen and also to digest such a substrate as hemoglobin, the ratios of these activities reported from various laboratories would be expected to be similar. Such does not appear to be the case.

The venom proteases tend to be of the tryptic type in reference to their pH optima and peptide substrate specificities, but are not affected by trypsin inhibitors. While the venoms show no chymotryptic activity, most of them destroy bradykinin, which is one of the functions of the latter enzyme as opposed to that of trypsin, which is capable only of forming this substance. The marked ability of the venom of *A. contortrix* to release bradykinin coupled with its failure to effect destruction strongly suggests the use of such venom in work on the preparation of this pharmacologically active agent. The commonly used trypsin and *B. jararaca* venom have several shortcomings. Trypsin is difficult to obtain free from chymotryp-

sin and is also markedly inhibited by serum antitrypsin. The *B. jararaca* venom, in addition to forming, also destroys bradykinin and hence gives lower yields. Furthermore, its use necessitates rather exacting experimental conditions.

The marked differences in the action of venom proteases and of trypsin on fibrinogen and fibrin are of interest in view of the reports of the tryptic-like actions both of thrombin (15) and of plasmin (23). If the clotting of fibrinogen is actually due to splitting of this molecule at the arginine peptide bond, it tends to produce a protein much less susceptible to hydrolysis by both trypsin and snake venom proteases. In any event the proteolysis of fibrinogen and of fibrin by venoms, trypsin, thrombin, and plasmin would appear to exhibit unique differences.

Owing in part to the complexity of the snake venom system, a more intimate knowledge of their proteases must be gained from work with purified enzymes. It is suggested that this should be an early goal of workers in this area.

The authors are indebted to Professor J. M. Gonçalves for much helpful discussion during the course of this work. One of us (H. F. D.) wishes to take this opportunity to express his gratitude for the hospitality of this laboratory during the tenure of a visiting professorship.

SUMMARY

The proteolytic activities of fifteen snake venoms on various substrates have been studied. Ethylenediaminetetraacetate, while strongly inhibiting the digestion of hemoglobin, does not affect the splitting of benzoyl-L-arginine ethyl ester. Ovomucoid and trypsin soy bean inhibitor have no inhibitor action on either reaction. Like trypsin, the venoms digest fibrinogen far more rapidly than fibrin.

All of the venoms liberate bradykinin from a serum globulin fraction. With the exception of *Agkistrodon piscivorus piscivorus* and *Agkistrodon contortrix* the venoms have the chymotryptic-like effect of destroying this substance. However, no appreciable action on acetyl-L-tyrosine ethyl ester is shown by any of the venoms.

No definite relations for the various proteolytic activities of a given venom are usually apparent.

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PARTIAL PURIFICATION OF PORCINE LIVER URICASE

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Uricase, the enzyme which oxidizes uric acid to allantoin, has been prepared from both porcine liver and bovine kidney in highly purified form (1–6). Davidson (1) and Holmberg (2), using acetone-dried porcine liver, reported preparations with Q_{O_2} (protein) of approximately 6000. Altman *et al.* (4) reported preparations from bovine kidney with a Q_{O_2} (protein) of 450, and Leone (5) prepared a material from the same tissue with a Q_{O_2} (protein) of approximately 700. The methods varied in their complexity and yields. A simple and reproducible new method for the preparation of porcine liver uricase was developed and is described in this report. This procedure was based on the early removal of water- and salt-soluble proteins, including nucleoproteins. Since preliminary experiments showed that uricase had solubility properties similar to some of the nucleoproteins, the removal of pentose nucleoprotein and deoxypentose nucleoprotein from the tissue, before the extraction of the enzyme, was indicated. In this method a soluble, stable enzyme preparation free of nucleoproteins is obtained. The enzyme has been purified 300-fold over an alkaline extract of whole liver in good yields with a final Q_{O_2} (protein) of 1200. Cysteine increases the rate of oxidation of uric acid by uricase (3) and the rate is dependent on the concentration of the activator.

EXPERIMENTAL

Measurement of Enzyme Activity

Uricase activity is determined at each step in the procedure by the Warburg manometric method at 37° (1, 2, 7, 8). The reaction chamber contained 40 μmoles of uric acid as lithium urate in 1.0 ml.,[1] 0.5 to 1.0 ml. of 0.1 M glycine buffer at pH 9.3, 0 to 1.5 ml. of enzyme in sodium carbonate (or sodium carbonate-glycine buffer) at pH 9.6 to 10.0, and distilled water to a total volume of 3.0 ml. The enzyme solution was always the last reagent added. The center well contained 0.2 ml. of 20 per cent KOH and a small strip of fluted filter paper. The gas phase was air. The mixture

[1] 672 mg. of uric acid are dissolved in 35 ml. of boiling 0.12 N LiOH and the solution is diluted to 100 ml. with distilled water.

was equilibrated for 5 minutes. The reaction was carried out for 60 minutes with readings taken every 10 minutes. All samples were run in duplicate.

The enzyme concentration was adjusted to give an oxygen uptake between 50 and 200 c.mm. per hour. The average oxygen uptake between 20 and 40 minutes, which was always on the linear portion of the curve, was taken to represent the average rate of the reaction. This value was used to calculate the specific enzyme activity. Under the conditions of these experiments, the enzyme activity, Q_{O_2} (protein), is defined as the microliters of oxygen consumed per mg. of protein per hour. Protein concentration was determined by a modification of the biuret method of Robinson and Hogden (9, 10), with crystalline bovine albumin as the protein standard.

Keilin and Hartree (7) found the pH optimum for uricase activity to be at 9.25. The activity at pH 7.4 was found to be approximately 75 per cent of that at pH 9.25 (extrapolated from the curve). In the development of Steps 1 to 3, the uricase activity was determined at pH 7.4 with 0.5 ml. of 0.48 M phosphate buffer at pH 7.4 in the assay system instead of the glycine buffer. In the development of Step 4, the uricase activity was determined at pH 8.4 with 0.5 ml. of Palitzsch's borate buffer at pH 8.4. At pH 7.4, the uricase extract and the uricase fraction PI[2] showed 83 per cent of the standard activity. Crystalline bovine liver catalase added to the assay system either with the uricase extract or with PI did not influence the oxygen uptake.

Purification

Step 1. Extraction of Impurities from Porcine Liver with Water

To each kilo of fresh, frozen comminuted or ground porcine liver are added 3 liters of ice-cold distilled water; the mixture is stirred overnight at 1°. The residue is removed by centrifugation at 4000 r.p.m. for 1 hour at 1° in the International refrigerated centrifuge, PR-1, and held for Step 2. The water extract, pH 6.2 to 6.4, contained 25 to 35 mg. of protein per ml. with a Q_{O_2} (protein) of 0 to 1. This solution, containing the bulk of the water-soluble protein and non-protein impurities, was discarded. The extract contained large amounts of pentose nucleoproteins and small amounts of deoxypentose nucleoproteins. The diphenylamine reaction (11) for deoxypentose and the orcinol reaction (12) for pentose were used semiquantitatively for the detection of nucleoprotein.

[2] The designations PI, PII, and PIII refer to the fractions obtained on the first, second, and third precipitation of the enzyme, respectively.

Step 2. Extraction of Impurities from Water-Washed Tissue with 0.045 M Phosphate Buffer at pH 7.7

The residue from the water wash is suspended in 3 volumes (based on original weight of tissue) of ice-cold 0.045 M phosphate buffer at pH 7.7 (6 volumes of Na_2HPO_4 and 1 volume of KH_2PO_4) and stirred overnight at 1°. The residue is removed by centrifugation at 4000 r.p.m. for 1 hour at 1° and held for Step 3. The phosphate extract, pH 7.2, containing 18 to 23 mg. of protein per ml. with a Q_{O_2} (protein) of 1 to 2, was discarded. Increasing the ionic strength of the phosphate buffer increases greatly the solubility of the uricase with very little increase in protein extracted. This extraction can be carried out from pH 7.0 to 7.4 and removes the residual pentose nucleoprotein and the bulk of the deoxypentose nucleoprotein.

Step 3. Extraction of Impurities from Phosphate Residue with 1 M Sodium Chloride

The residue from the phosphate wash is suspended in 3 volumes (based on original weight of tissue) of ice-cold 1 M NaCl and stirred overnight at 1°. The residue is removed by centrifugation at 4000 r.p.m. for 1 hour at 1° and held for Step 4. The sodium chloride extract contained 5 to 10 mg. of protein per ml. with a Q_{O_2} (protein) of 0 to 4. It contained a small amount of pentose nucleoprotein and a trace of deoxypentose nucleoprotein and was discarded. This step was used to remove the last traces of deoxypentose nucleoprotein. At lower ionic strengths not as much nucleoprotein and protein was extracted. With increasing ionic strength additional protein was not extracted. There was negligible extraction of activity from ionic strength 0.05 to 3.0.

Step 4. Extraction of Impurities from Sodium Chloride Residue with 0.05 M Sørensen Borate Buffer, pH 8.7

The residue from the sodium chloride wash is suspended in 3 volumes (based on original weight of tissue) of ice-cold 0.05 M Sørensen borate buffer at pH 8.7 (2.43 volumes of 0.05 M sodium borate and 1 volume of 0.1 N HCl) and stirred overnight at 1°. The residue is removed by centrifugation at 4000 r.p.m. for 1 hour at 1° and held for Step 5. The borate extract, pH 8.3 to 8.4, containing 5 to 10 mg. of protein per ml. with a Q_{O_2} (protein) of 0 to 4, was discarded. There was still a trace of pentose nucleoprotein. In addition to the removal of the last traces of nucleoprotein, non-uricase alkali-soluble proteins were also extracted in this step, leaving the bulk of the uricase insoluble. This extraction can be carried

out between pH 8.0 and 8.6. Small amounts of activity are extracted above pH 8.6.

Step 5. Extraction of Uricase from Borate Residue with 0.27 M Sodium Carbonate-0.10 M Glycine Buffer, pH 10.2

The residue from the borate wash is suspended in 3 volumes (based on original weight of tissue) of ice-cold 0.27 M Na_2CO_3-0.10 M glycine buffer at pH 10.2 and stirred overnight at 1°. The residue is removed by centrifugation at 4000 r.p.m. for 1 hour at 1° and discarded. The uricase extract, pH 10, contains 2 to 4 mg. of protein per ml. with a Q_{O_2} (protein) of 24 to 35. The yield was 46 to 54 per cent with a 6- to 11-fold purification over an alkaline extract, pH 10, of whole liver, which contained 50 mg. of protein per ml. with a Q_{O_2} (protein) of 4. The uricase extract occasionally contained a trace of pentose nucleoprotein but no deoxypentose nucleoprotein. Preliminary experiments showed that maximal uricase activity can be extracted at pH 10. Lower concentrations of sodium carbonate, which gave lower pH values, extracted less activity. Glycine increased the extractability of the uricase by sodium carbonate. Extraction with NaOH at pH 11 gave no activity. Table I summarizes the semiquantitative tests for pentose and deoxypentose nucleoproteins on the extracts obtained at Steps 1 to 5. The four washes removed over 90 per cent of protein and approximately 50 per cent of the uricase activity found in an alkaline extract, pH 10, of whole liver. In certain experiments in which practically no uricase was extracted in Steps 1 to 4, the final carbonate-glycine extract still contained only 50 per cent of the total activity.

TABLE I

Semiquantitative Tests for Nucleoproteins Extracted at Various Steps

Step No.	Pentose nucleoprotein*	Deoxypentose nucleoprotein†
1	++++	+
2	++++	++++
3	++	+
		−
4	+	−
5	+	−
	−	

* Orcinol reaction (12) for pentose (ribose).

† Diphenylamine reaction (11) for deoxypentose (deoxyribose).

Step 6. Precipitation of Uricase PI from Sodium Carbonate-Glycine Extract with Ethanol under Controlled Conditions of pH, Ionic Strength, Protein Concentration, and Temperature

The sodium carbonate-glycine extract of uricase is adjusted to pH 7.3 ± 0.2 with 1 N HCl. 95 per cent ethanol (3A[3]) at −25° is added to a final concentration of 40 per cent ethanol at −5°. The final conditions are protein concentration, 1.7 ± 0.6 mg. per ml.; pH 7.3 ± 0.2; ionic strength, 0.26 ± 0.02; ethanol concentration, 40 per cent; temperature, −5°. The mixture was held at −5° overnight. The precipitate is removed by centrifugation at 4000 r.p.m. for 30 minutes at −5° and then suspended in 10/25 of the original extract volume of ice-cold 0.02 M Na_2CO_3 at 1°. The suspension is stirred overnight and clarified at 4000 r.p.m. for 1 hour at 1°. The uricase PI solution, pH 10, contains 1 to 2 mg. of protein per ml. with a Q_{O_2} (protein) of 90 to 210. The step yield was 70 to 80 per cent and the over-all yield was 37 to 43 per cent, with a 30- to 35-fold purification. Some of the enzyme precipitated before the addition of ethanol. The enzyme can be quantitatively precipitated from the uricase extract at pH 5.2 ± 0.3 in the absence of ethanol but with only a 2-fold purification. Apparently the high concentration of ethanol in this step denatures some of the non-uricase proteins. The purpose of this step was to concentrate the enzyme before further purification.

Step 7. Further Purification with Ethanol-Water Mixtures under Controlled Conditions, Uricase PII

The solution of uricase PI in 0.02 M Na_2CO_3 at pH 9.9 ± 0.2 is adjusted with solid sodium chloride to 0.26 M. 95 per cent ethanol (3A) at −25° is added to a final concentration of 40 per cent ethanol at −5°. The final conditions are protein concentration, 0.9 ± 0.3 mg. per ml.; pH 9.9 ± 0.2; ionic strength, 0.15; ethanol concentration, 40 per cent; temperature, −5°. The mixture was held overnight at −5°. The precipitate is removed by centrifugation at 4000 r.p.m. for 30 minutes at −5° and is discarded. The supernatant solution contains all of the uricase and approximately 14 per cent of the protein. The precipitation of impurities by ethanol at this point is affected by ionic strength. Decreasing the ionic strength below 0.10 results in a decrease in protein precipitated. Increasing the ionic strength above 0.10 up to 0.40 has no effect on either increasing the precipitation of protein impurities or precipitating the enzyme. The supernatant solution is adjusted to pH 6.3 ± 0.2 with 1 N HCl to precipitate

[3] Denatured alcohol formula No. 3A, ethanol 190 proof containing 5 per cent commercially pure methanol.

the uricase. The mixture was held overnight at −5°. The precipitate is removed by centrifugation at 4000 r.p.m. for 1 hour at −5° and then suspended in 1/25 of the original extract volume of ice-cold 0.01 M Na_2CO_3 at 1°. The precipitate is readily and completely soluble. The uricase PII solution, pH 10, contains 1 to 2 mg. of protein per ml. with a Q_{O_2} (protein) of 1150 to 1250. The step yield was 90 to 100 per cent and the over-all yield was 35 to 38 per cent with a 288- to 313-fold purification. The precipitation of uricase PII from the 40 per cent ethanol solution at pH 10 gave optimal results at pH 6.3 ± 0.2. The yields are not as good at pH 6.5 to 7.5. At values below pH 6.1, there is some indication of decrease in solubility of the enzyme although the yields are good. Uricase can also

TABLE II

Summary of Uricase Purification Procedure

Step No.	Fraction	Protein	Q_{O_2} (protein)	Over-all yield	Purification factor*
		mg. per ml.		*per cent*	
	Alkaline tissue extract	50	4	100	
1		25–30	0– 1	0–15	
2		18–23	1– 2	8–23	
3		5–10	0– 4	0–15	
4		5–10	0– 4	0–15	
5	Extract	2– 4	25– 45	46–54	6– 11
6	PI	1– 2	120– 140	37–43	30– 35
7	PII	1– 2	1150–1250	35–38	288–313

* Purification factor = (Q_{O_2} (protein) of fraction)/(Q_{O_2} (protein) of alkaline tissue extract).

be precipitated from PI at pH 5.2 ± 0.3 in the absence of ethanol but with no purification. All of the proteins apparently have the same solubility properties. The results of the entire isolation procedure are summarized in Table II.

A uricase PII preparation with a Q_{O_2} (protein) of 1200 at 100 γ of protein per ml., pH 8.6, gave an optical density of 0.164 at 293 mμ. 100 γ of enzyme protein will decompose 15 γ of uric acid per minute.

Further Purification

Uricase PII can be further purified. Preliminary experiments have shown that uricase PIII can be precipitated at pH 8.6 ± 0.3, protein concentration of 1.2 ± 0.4 mg. per ml., ionic strength of 0.12 ± 0.04, and ethanol concentration of 20 per cent at −5°. The preparations gave a Q_{O_2} (protein) of 2000 to 2800 which was approximately a 2-fold purification over PII with a yield of 70 to 90 per cent.

Stability

Uricase extract, PI, and PII can be stored at 1° for several months without loss in activity. In addition, uricase PII can be dialyzed at pH 10 and lyophilized without loss in activity.

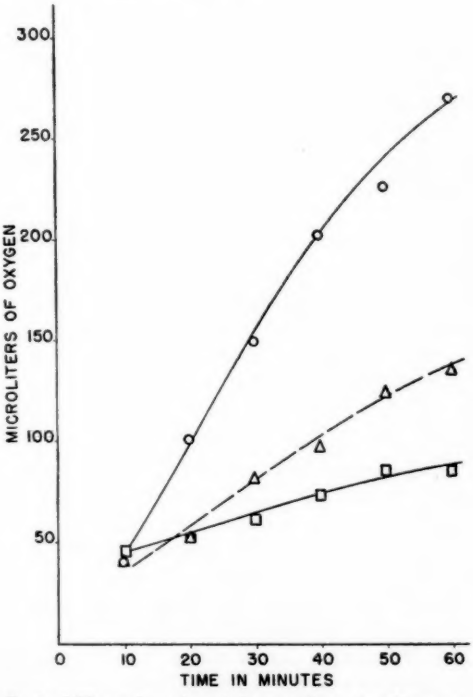

FIG. 1. The effect of 30 μmoles of cysteine on the rate of oxygen consumption in the oxidation of uric acid by uricase PI. ○, uricase PI plus uric acid plus cysteine; △, uricase PI plus uric acid; □, uricase PI plus cysteine.

Ion Effect on Solubility Properties

Uricase PI or PII can be dissolved in Na_2CO_3 at pH 10 and in sodium citrate at pH 7.4. The choice of solvent used in the isolation was influenced by the procedure which was planned for the next step. The enzyme can be completely dissolved in 0.15 M sodium citrate at pH 7.4 and at an ionic strength of 0.9; lower concentrations of salt did not dissolve the enzyme completely. In the extraction of impurities from the tissue at Step 2, it was found that high concentrations of phosphate buffer (ionic strength, 0.3 to 0.9) at pH 7.2 to 7.4 extracted large amounts of uricase.

In Step 3, high concentrations of sodium chloride (ionic strength, 0.3 to 3.0) at pH 7.0 extracted very little enzyme. The solubility of uricase at neutral pH in salts at high ionic strengths seems to be influenced by the valence of the anion. The anion with the highest valence, citrate ion, was the most effective in making the enzyme soluble.

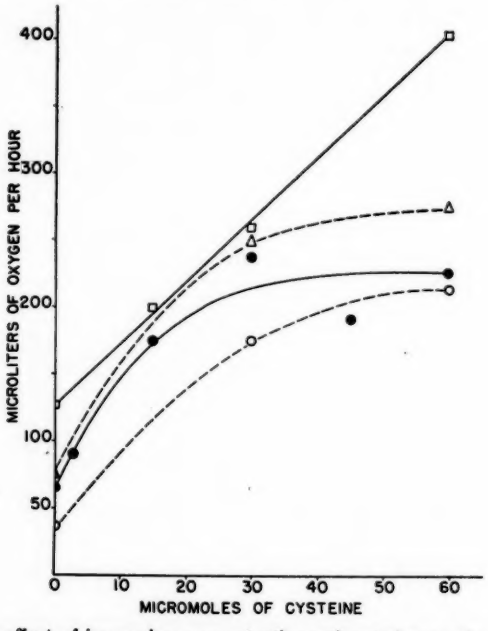

FIG. 2. The effect of increasing concentrations of cysteine on the rate of oxygen consumption in the oxidation of uric acid by three different concentrations of uricase PI and one concentration of uricase PII. Uricase PI, ○, 36 c.mm. per hour; △, 75 c.mm. per hour; □, 126 c.mm. per hour; uricase PII, ●, 63 c.mm. per hour. Q_{O_2} values plotted after subtraction of cysteine blank.

Activation Studies with Cysteine

Davidson (13) reported that thioacetic and dithioacetic acids accelerated the uptake of oxygen in the oxidation of uric acid by uricase. Scheer and Scheer (3) showed that cysteine increased the rate of oxygen uptake but found that the rate is independent of the amount of cysteine added. Glutathione showed a similar effect. The cysteine "activating" effect was studied with uricase PI and PII. Solutions of cysteine hydrochloride adjusted to pH 7.0 with sodium hydroxide were prepared at the time of assay and introduced into the reaction chamber before assay. The effect

of 30 μmoles of cysteine on uricase PI is shown in Fig. 1. The cysteine-enzyme-uric acid system showed a rate of 318 c.mm. per hour as compared to the enzyme-uric acid system which showed a rate of 141 c.mm. per hour. The cysteine-enzyme system showed a rate of 69 c.mm. per hour. There is a definite effect of cysteine on the rate of oxygen consumption. Cysteine is oxidized very slowly by the uricase preparations in the absence of uric acid. The effect of increasing concentrations of cysteine at three different concentrations of uricase PI is presented in Fig. 2. There is a definite increased rate of oxidation at the levels of uricase activity studied. With increasing concentrations of cysteine, uricase PII at one level gave similar results.

SUMMARY

A simple and reproducible new method for the preparation of highly purified porcine liver uricase is described. By this method a soluble, stable enzyme preparation free of nucleoproteins, nucleic acids, and water- and salt-soluble proteins is obtained. The enzyme has been purified 300-fold over an alkaline extract of whole liver in yields of approximately 36 per cent with a Q_{O_2} (protein) of 1200. Preliminary experiments on the further purification of the enzyme gave preparations with a Q_{O_2} (protein) of 2000 to 2800. The enzyme can be stored at pH 10 for several months at 1° without loss in activity. It can also be dialyzed at pH 10 and lyophilized without loss in activity. The effect on the solubility of uricase at pH 7.4 by salts at high ionic strengths is influenced by the valence of the anion. The anion with the highest valence studied, the citrate ion, was the most effective in making the enzyme soluble. Cysteine increased the rate of oxygen uptake during the oxidation of uric acid by uricase. The increase in the rate is dependent on the concentration of activator.

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STUDIES OF SOME PRECURSORS OF PYRIMIDINES*

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The utilization of ureidosuccinic and orotic acids for pyrimidine formation has been well established (2–5). Lieberman and Kornberg (6) have reported the interconversion of these compounds via dihydroorotic acid by a cell-free extract of an organism tentatively named *Zymobacterium oroticum*, suggesting that this is the mechanism utilized for the conversion of ureidosuccinic acid to pyrimidines.

The demonstration that the carbon of carbon dioxide enters position 2 of pyrimidines (7) and that a structural analogue of ureidosuccinic acid, argininosuccinic acid, is an intermediate in the formation of urea (8, 9) has suggested the possible involvement of the urea cycle in pyrimidine formation.

The data to be given indicate that (*a*) the mammalian organism also is capable of converting ureidosuccinic acid to orotic acid by way of dihydroorotic acid, (*b*) the urea cycle appears not to function appreciably in the formation of pyrimidines in rats, and (*c*) aminofumaric acid diamide is an efficient precursor of nucleic acid uracil in slices of rat liver.

Preparation of Labeled Compounds

Orotic acid-2-C^{14} *and ureidosuccinic acid-C*^{14}, labeled in the ureide carbon, were prepared by the method of Nyc and Mitchell (10) from urea-C^{14}.

Dihydroorotic acid-2-C^{14} was prepared from orotic acid-2-C^{14} by hydrogenation as follows: 50 mg. of orotic acid were refluxed in 40 ml. of glacial acetic acid until solution was effected. The solution was cooled to room temperature, and 70 mg. of Adams' catalyst were added. Hy-

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drogenation was conducted in a Parr apparatus with continuous shaking for 20 hours at 50 pounds pressure. Both the amount of catalyst and time of shaking appeared to be important. After hydrogenation, the solution was filtered and evaporated under reduced pressure at 40° to a volume of 3 ml. 15 ml. of pentane were added, and the suspension was centrifuged. The precipitate was repeatedly washed with pentane until free of the odor of acetic acid. It was then dissolved in 5 ml. of hot water and filtered. 3 volumes of absolute ethanol were added to the filtrate, and the dihydroorotic acid was removed by filtration. The product was washed with ether and dried at 100°. The yield was 48 mg. and the product decomposed at 265–267°. This compound is biologically active in the *Z. oroticum* system of Lieberman and Kornberg[1] and the *Lactobacillus bulgaricus* 09 system of Wright *et al.*,[2] in contrast to the material prepared by the method of Bachstez and Cavallini (11), which was not utilized in either system. Another method of synthesis has recently been reported by Miller, Gordon, and Engelhardt (12).

Citrulline-C^{14}-N^{15} labeled in the carbamyl group was prepared by the following procedure. Urea-N^{15}, labeled in 1 nitrogen atom, was prepared from $N^{15}H_4Cl$ by the method of Williams and Ronzio (13). This was then mixed with urea-C^{14} obtained from Tracerlab, Inc. This mixture was recrystallized from acetone and used to make L-citrulline from L-ornithine and urea by the method of Kurtz (14). The product melted at 220–221° (uncorrected) and contained 23.4 per cent nitrogen (calculated 24.0 per cent). Kurtz reported a melting point of 226°. The preparation gave only one ninhydrin spot with the R_F of citrulline following chromatography in phenol-water.

Aminofumaric acid diamide-3-C^{14} was prepared as follows: Sodium acetate-2-C^{14} was converted to its ethyl ester by the method of Sakami, Evans, and Gurin (15). This was in turn converted to diethyl sodiooxalacetate-3-C^{14} by the method of Wislicenus (16). The free ester was obtained from the salt by shaking with 5 N HCl and ether until all of the solid dissolved, drying the ether layer over $MgSO_4$, and removing the ether. This compound was converted to diethyl chlorofumarate-3-C^{14} by the method of Michael and Schulthess (17). This in turn was allowed to react with aqueous ammonia, as described by Perkin (18), to give aminofumaric acid diamide 3-C^{14}. This compound contained 32.5 per cent nitrogen, which is identical with the theoretical value. When the compound was recrystallized from methanol, it decomposed at 206–208° (uncorrected). Thomas-Mamert (19) records a value of 190–195°. Although the method of synthesis was straightforward, further points of evidence concerning the structure of the compound are given below.

[1] Kornberg, A., personal communication.
[2] Miller, C. S., personal communication.

20 mg. of the aminofumaric acid diamide were catalytically reduced in glacial acetic acid solution with an excess of Adams' catalyst for 2 hours at atmospheric pressure. The resulting material was hydrolyzed by boiling with acetic and hydrochloric acids, evaporated to dryness, and dissolved in 5 ml. of water. The absorption of ultraviolet light by this solution, both acid and alkaline, was reduced to negligible amounts at 295 mμ. A portion of the solution was chromatographed on Whatman No. 1 paper alongside DL-aspartic acid with a pyridine-water (65:35) solution. Both materials yielded similar purple spots having the same R_F (0.41). These were developed by spraying with ninhydrin solution and heating. It appeared that aminofumaric acid diamide formed aspartic acid by reduction and hydrolysis.

An amino nitrogen determination by Van Slyke's method was carried out on the diamide. Calculated results showed that about 60 per cent of 1 nitrogen atom had reacted in 5 minutes at 25°, about 80 per cent in 10 minutes, and about 130 per cent in 30 and 60 minutes. These results are considered to be typical of such a compound, which is probably in the imino form to a considerable extent in a solution acid with acetic acid.

Ultraviolet Absorption of Aminofumaric Acid Diamide[3]—In order to identify and estimate the amount of this compound, the molecular extinctions and absorption curves were determined in the ultraviolet region at various pH values (see Fig. 1). It may be seen that in neutral and alkaline solutions amino fumaric acid diamide had characteristic absorption, with a good maximum at about 295 mμ. The molecular extinction at pH 9 and 294 mμ was 9600. At pH 5 and below, the compound underwent a more or less gradual rearrangement and the absorption of ultraviolet light almost disappeared.

The rate of change in optical density at 295 mμ in acid solution is shown in Fig. 2. At pH 5, the optical density had diminished to 80 per cent of that observed in alkaline solution by the time (after 3 minutes) the first reading was made. At the end of 60 minutes the optical density was less than 20 per cent of the original. At pH 1, the reaction was so rapid that practically all of the absorption at 295 mμ had disappeared by the time the first reading was made. At the end of 60 minutes, all solutions were made slightly alkaline and the optical densities at 295 mμ returned to the original value.

Isolation and Purification of Purines, Pyrimidines, and Protein. Method 1—At the completion of the incubation in the homogenate experiments, in which the formation of orotic acid was studied, 19 μmoles of carrier orotic acid were added and the homogenate transferred to a 12 ml. centrifuge tube with the aid of 5 ml. of water. The tube was heated in a water bath at

[3] These data were obtained by Mr. Thomas A. Courtenay and Mrs. Marjorie Allison Smith.

100° for 5 minutes to coagulate the protein. After centrifuging, the precipitate was washed twice with a total of 1.5 ml. of water. The supernatant fluid and washings were put on a Dowex 1 column (chloride form) 7.5 × 0.8 cm. The column was then washed with 750 ml. of 0.002 N HCl,

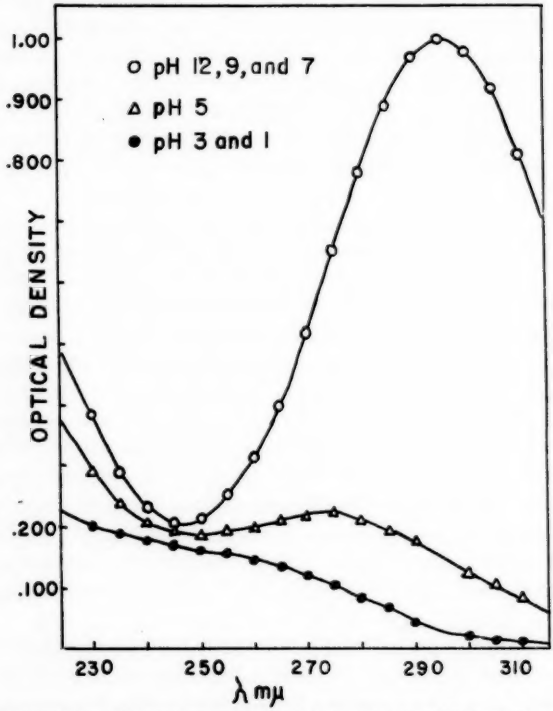

FIG. 1. Ultraviolet absorption curves of 1 × 10^{-4} M solutions of aminofumaric acid diamide at various pH values. The curves obtained at pH 12, 9, and 7 were so similar that no attempt was made to plot them separately. In acid solutions, in which the optical densities changed with time, the readings on the left of the chart were made first and the others in order, without reference to the time which elapsed.

which removed any soluble protein, hypoxanthine, and most of the ureidosuccinic acid or dihydroorotic acid. The orotic acid was then eluted with 75 ml. of 0.10 N HCl. To free the orotic acid from traces of ureidosuccinic acid or dihydroorotic acid, the orotic acid fraction was evaporated to dryness at 40°, taken up in water, and rerun on a Dowex 1 column (chloride form), the dimensions of which were 14.0 × 0.8 cm. The contaminants were eluted with 100 ml. of 0.008 N HCl, whereas the orotic acid required 650 to 800 ml. of the acid for elution.

Method 2—In the experiment demonstrating the conversion of ureidosuccinic acid to dihydroorotic acid, 12.5 μmoles of non-radioactive dihydroorotic acid were added as a bank at the beginning of the incubation. The protein-free supernatant fluid and washings were put on the Dowex 1 column as described above. The 0.002 N HCl fraction was evaporated to

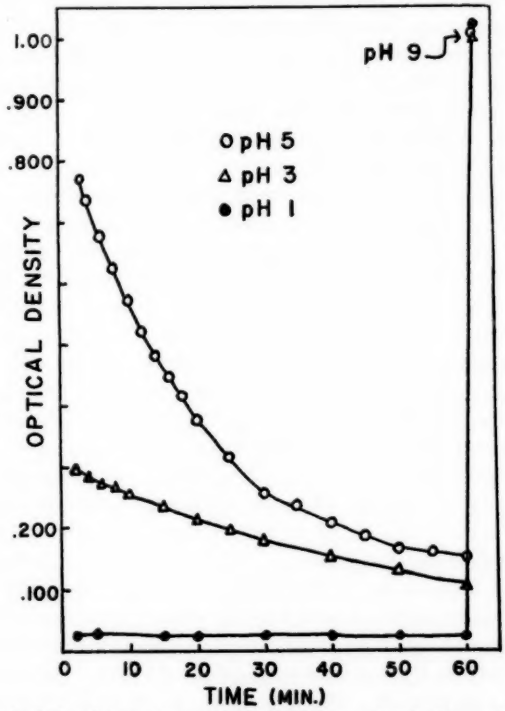

FIG. 2. Optical densities at wave-length 295 mμ of solutions of aminofumaric acid diamide at various pH values, showing how the densities vary with time of standing. The plotting in the upper right corner represents the density of each solution when it was made slightly alkaline.

dryness at 40°, taken up in water, and chromatographed on Whatman No. 1 filter paper for 14 hours in 4:1:1 *n*-BuOH-HOAc-H_2O. In this solvent dihydroorotic acid has an R_F of 0.20, while that of ureidosuccinic acid is 0.43. A thin strip was cut from the edge of each paper, and the bands were made visible by the use of phenol red spray or by the formation of mercury salts. The central portion of the dihydroorotic acid band was eluted with water and rechromatographed three additional times in this solvent.

Method 3—Tissue nucleic acids and protein were isolated by the method

of LePage and Heidelberger (20). In the experiment with L-citrulline, the purines and pyrimidines were also isolated by the methods described by these authors with a few modifications. The pyrimidine nucleotide fraction was hydrolyzed with 72 per cent $HClO_4$, and the uracil was finally purified on Dowex 1 (hydroxide form). The uracil was eluted with 0.003 N HCl, and the elution fluid containing the uracil was free of chloride ions. Each of the compounds was then rerun on its respective resin column at least once, in some cases twice, until it was considered to be pure. In all of the work described, the criteria employed for purity were the symmetry of the elution curves and the ratios of the optical densities at the following wave-lengths (millimicrons) at the appropriate pH: 290/260, 280/260, 250/260, and 230/245 (21, 22).

Method 4—For the experiments with slices of regenerating rat liver, the nucleic acids, isolated as described above (20), were hydrolyzed in 1 N HCl and the hydrolysate chromatographed on paper in *t*-BuOH-HCl (23). The pyrimidine nucleotide bands were eluted with water, evaporated to dryness in 15 ml. test-tubes at 40°, and the nucleotides hydrolyzed with 72 per cent $HClO_4$. The adenine and cytosine were purified on Dowex 50 and the uracil on Dowex 1 (hydroxide form). After each of the compounds was run through a complete purification procedure, a portion was utilized for the determination of the specific activity. The remainder was chromatographed on paper in *n*-BuOH-urea (24), then rerun on a resin column, and the specific activity redetermined. The values in Table II are the mean values, and the standard deviations encountered were within the counting error.

Isolation of Urinary Ammonia and Urinary Urea Nitrogen and Carbon—5 ml. of pooled urine were brought to pH 9 with saturated K_2CO_3 and aerated with air freed of CO_2 and NH_3 into a solution of boric acid containing the indicator mixture commonly employed for Kjeldahl nitrogen analysis. The collection vessels were changed at 30 minute intervals until no more ammonia came over. The pH was then adjusted to 5 with ammonia-free HCl. 6 ml. of 0.1 M acetate buffer at pH 4.8 were added, and the flask was aerated with air freed of carbon dioxide into saturated $Ba(OH)_2$ until no further carbon dioxide was recovered. 120 mg. of urease were then added, and the flask was heated in a water bath at 39° for 30 minutes. The carbon dioxide derived from the urea was then removed by aeration as described. The pH was again changed to 9 and the ammonia removed by aeration. Citrulline was unaffected by these conditions.

Collection of Respiratory Carbon Dioxide—These samples were collected by the method described by Delluva and Wilson (25) 30 minutes after each injection of citrulline. The same rat was used for the collections throughout the experiment.

N^{15} Collection—Purified compounds were subjected to Kjeldahl digestion for 12 hours. The ammonia formed was converted to nitrogen as described by Rittenberg (26).

Determination of Specific Activities—The concentrations of the fractions from the resins were determined with the aid of the extinction coefficients of the pyrimidines reported by Shugar and Fox (21) and the purine values of Kerr *et al.* (27). These fractions were then transferred to aluminum planchets as described by Weed and Wilson (3). All radioactivity determinations were made on samples of 400 to 600 γ to a standard error of 5 per cent except in the one case noted. In the homogenate experiments in which a bank of orotic acid was employed, the purified material from the resin column was divided into four consecutive portions and the specific activity of each determined. This was to test the purity of the compound with respect to the possible presence of radioactive contaminants having no ultraviolet absorption. In all cases the deviations of specific activity were within the counting error.

Results

Conversion of Ureidosuccinic Acid and Dihydroorotic Acid to Orotic Acid by Homogenates of Rat Liver—The precursor being studied was incubated with the homogenate for varying periods of time. The rates of conversion to orotic acid are shown in Fig. 3. The curves indicate that dihydroorotic acid is converted to orotic acid much more readily than is ureidosuccinic acid. This is compatible with the concept that ureidosuccinic acid is converted to orotic acid via dihydroorotic acid.

To establish this point further, a similar homogenate was incubated for 45 minutes with 12.5 μmoles of radioactive DL-ureidosuccinic acid and a bank of 12.5 μmoles of DL-dihydroorotic acid. Dihydroorotic acid was isolated, purified as described under Method 2, and its radioactivity determined. The dihydroorotic acid band of the final chromatogram contained 2000 c.p.m., while the region normally occupied by ureidosuccinic acid had no activity. These results indicate that the rat liver homogenate is capable of carrying out the following conversion,

Ureidosuccinic acid → dihydroorotic acid → orotic acid

Rôle of Urea Cycle in Pyrimidine Formation—An experiment was carried out with rats to explore the possibility that the urea cycle is concerned with pyrimidine formation. Six male Wistar rats weighing approximately 110 gm. each were injected intraperitoneally every 4 hours for 3 days (with the exception of the sixth 4 hour period of each day) with 1 ml. of physiological saline containing 3 mg. of L-citrulline labeled with C^{14} and N^{15} in the carbamyl moiety, in which the specific activity was 8.75×10^6

c.p.m. per mg. of C and the N contained 24.21 atom per cent excess N^{15}. 30 minutes after each injection a sample of the expired carbon dioxide was collected and the specific activity determined as $BaCO_3$. The values obtained ranged from 143 to 1710 c.p.m. per mg. of C. The animals were sacrificed by cervical dislocation 14 hours after the last injection and the livers, spleens, kidneys, testes, and small intestines pooled. The purines and pyrimidines and protein were isolated and purified as described under

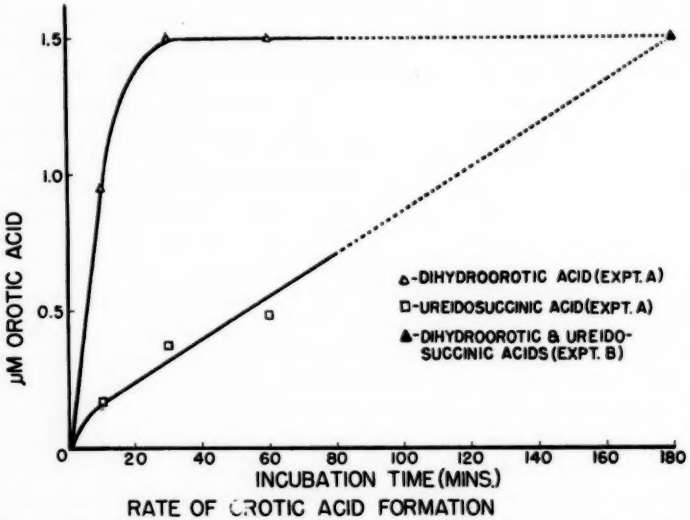

FIG. 3. Vessels containing 3.0 ml. of 2:1 rat liver homogenate in the medium of Schulman *et al.* (28) and 12.5 μmoles of a DL mixture of the radioactive precursor in a final volume of 3.5 ml. were incubated at 25°. For the 10, 30, and 60 minute incubations, aliquots of a single homogenate were used, while the 180 minute incubations utilized aliquots of a second homogenate.

Method 3. Table I shows the values obtained for the compounds that were isolated and analyzed. The fact that the carbamyl group of the citrulline was utilized intact for the formation of urea and not for the formation of purines or pyrimidines[4] can be seen readily by comparing the utilization

[4] Schulman and Badger (29) have shown that, while no appreciable amount of citrulline enters the pyrimidines of rats, it does contribute directly to the pyrimidines of pigeons, in which little urea is formed and excreted. Heinrich, Dewey, and Kidder (30) administered citrulline labeled with C^{14} in the carbamyl group to rats and a chick and observed no incorporation. They obtained incorporation into uracil and cytosine when they used an arginine-requiring mutant of *Neurospora*. Smith and Stetten (31), studying incorporation of citrulline into orotic acid, obtained negative

of C^{14} and N^{15} in Table I. The utilization of these two elements in forming urinary urea was approximately the same and was similar on all 3 days. However, the utilization of both C^{14} and N^{15} in purines and pyrimidines was of an order that could best be explained by assuming that the isotopes come from pools of carbon dioxide and "ammonia" and not directly from citrulline.

Utilization of Orotic Acid, Ureidosuccinic Acid, and Aminofumaric Acid Diamide for Formation of Pyrimidines in Slices of Rat Liver (Table II)—In

TABLE I

Incorporation in Vivo of Isotope from L-*Citrulline into Various Compounds*

Compound	Atom per cent excess N^{15}	C.p.m. per mg. C	N^{15} utilization*	C^{14} utilization†
1st day urinary NH_3	0.078		0.32	
2nd " " "	0.091		0.38	
3rd " " "	0.051		0.21	
1st " " urea N‡ or C	0.085	32,600	0.35	0.37
2nd " " " "	0.095	39,100	0.39	0.45
3rd " " " "	0.075	24,200	0.31	0.28
Protein		560		0.0064
Uracil	0.020	145	0.083	0.0016
Cytosine	0.042	135	0.174	0.0015
Adenine	0.006	105	0.025	0.0012
Guanine	0.011	100	0.046	0.0012

* (Atom per cent excess N^{15} in compound × 100)/(atom per cent excess N^{15} in carbamyl N of citrulline).

† (Counts per minute per mg. of C in compound × 100)/(counts per minute per mg. of C in carbamyl C of citrulline).

‡ Urinary urea N values calculated as follows: (observed atom per cent excess N^{15}) × 2 − (urinary NH_3 excess N^{15} value for the same day).

the first experiment fifteen male and female Wistar rats, weighing approximately 90 gm. each, were subjected to partial hepatectomy by the method described by Crandall and Drabkin (33). After allowing 24 hours for partial liver regeneration, the rats were killed by cervical dislocation, the livers removed and sliced with a Riggs-Stadie slicer, and the slices pooled in a solution of cold Krebs-Ringer saline with added phosphate buffer (34). Alternate slices were distributed among three 250 ml. conical flasks, each containing a total of 50 ml. of medium and 75 μmoles of one of the three precursors. The total wet weight of tissue in each of the three

results with pigeon liver slices and positive results with rat liver slices. The explanation of these results is not obvious at present. Reichard (32) has reported that he was unable to obtain any conversion of citrulline to orotic acid by rat liver slices.

flasks was the same. All of the operations were carried out at 4°. The flasks were incubated at 37° for 4 hours and the purines and pyrimidines isolated and purified as described in Method 4.

TABLE II

Conversion of Orotic Acid, Ureidosuccinic Acid, and Aminofumaric Acid Diamide to Pyrimidines by Slices of Rat Liver

Precursor	Specific activity	Nucleic acid		C^{14} utilization*		Acid-soluble uracil, specific activity	C^{14} utilization,* acid-soluble uracil
		Uracil	Cytosine	Uracil	Cytosine		
		Specific activity					
Regenerating liver							
	c.p.m. per μmole	*c.p.m. per μmole*	*c.p.m. per μmole*			*c.p.m. per μmole*	
Orotic acid	4.1×10^4	209	106	0.51	0.26		
Ureidosuccinic acid	5.1×10^4	69	34	0.14	0.07		
Aminofumaric acid diamide	9.7×10^3	21	4.3†	0.22	0.04		
Ureidosuccinic acid	9.7×10^3	78		0.8		3034	31.2
Aminofumaric acid diamide	9.7×10^3	4.2		0.04		104	1.07
Normal liver							
Ureidosuccinic acid‡	9.7×10^3					2820	29.0
Aminofumaric acid diamide‡	9.7×10^3					55	0.58
Ureidosuccinic acid§	9.7×10^3					2420	25.0
Aminofumaric acid diamide§	9.7×10^3					208	2.1

* (Counts per minute per micromole of pyrimidine × 100)/(counts per minute per micromole of precursor).

† Counted to a standard error of 10 per cent.

‡ Rats 6 to 8 weeks old.

§ Rats 4 weeks old.

The last three experiments were carried out in the same way, except that the solutions were first run through a charcoal column (35) (Darco G-60 mixed with Celite, 6 × 1 cm.). After washing with water to remove salts and proteins, the nucleotides and uracil were eluted with ammonia in ethanol (concentrated NH_4OH 10, ethanol 25, water 65). The eluate was evaporated at about 40° under a current of air to a small volume and then chromatographed on paper, etc., as described in Method 4.

The third and fifth experiments of Table II demonstrate that aminofumaric acid diamide is a precursor of nucleic acid pyrimidines, although it is not as effective as ureidosuccinate. Mitchell and Houlahan (36) found that aminofumaric acid diamide was one-tenth as effective as uracil for the growth of a *Neurospora* mutant.

In the last three experiments the acid-soluble uracil was isolated. The incorporation of isotope of both precursors into this compound was far greater than into the uracil of nucleic acids. Hurlbert (37) reported that acid-soluble uracil had about twice the specific activity of the uracil of nuclear ribonucleic acid. We have found that the acid-soluble uracil may have 20 times the specific activity of the uracil of nucleic acid of whole liver. The isolated uracil has a specific activity of 25 per cent (or greater) of the ureidosuccinate, whereas with aminofumaric acid diamide the specific activity of uracil was only 0.5 to 2 per cent of that of the precursor.

The finding of uracil rather than uridylic acid in the acid-soluble fraction is probably due to the action of degradative enzymes on the acid-soluble uridylic acid under the experimental conditions employed.

It is of interest to record that neither orotic acid, ureidosuccinic acid, nor aminofumaric acid diamide enters adenine, although they are incorporated into the pyrimidines.

DISCUSSION

The results obtained with ureidosuccinic acid and dihydroorotic acid in homogenates of rat liver and the results with ureidosuccinic acid and orotic acid in slices of rat liver indicate that the mammalian organism can convert ureidosuccinic acid to orotic acid via dihydroorotic acid. Thus a metabolic conversion originally demonstrated in a bacterium may prove to be ubiquitous.

Because of the close structural similarity between ureidosuccinic acid and argininosuccinic acid, an intermediate in the urea cycle, it seemed possible to us that the urea cycle might function in the biosynthesis of pyrimidines. The possibility, in rats, seems to have been definitely excluded by the results of the experiment with doubly labeled citrulline. The labeled portion is the moiety which should be transferred intact to pyrimidines if they were formed via argininosuccinic, ureidosuccinic, dihydroorotic, and orotic acids. This was not the case. It may be calculated that the C^{14} activity of the pyrimidines was only approximately 1 per cent of that which would have been expected if the pyrimidines were formed via argininosuccinic acid.[5]

[5] By utilizing the known data concerning the amount of isotope administered, an estimate of 100 mg. per day per rat for normal urea output (three determinations on the experimental animals prior to injection gave values ranging from 120 to 175 mg. per day per rat), and the specific activity of the urinary urea, an estimate may be

These results may also be interpreted as indicating that urea *per se* is not utilized for pyrimidine formation. Although Barnes and Schoenheimer (38) studied the possible utilization of urea for purine formation, they did not isolate the pyrimidines, and this point has never been established.

It is of interest to note that the citrulline nitrogen is apparently more readily available for the formation of pyrimidines than of purines. Lagerkvist (39) has shown that in rats, two of the purine ring nitrogens and one of the pyrimidine ring nitrogens come from ammonia. This would suggest that in our experiments the N^{15} content of uracil and adenine should be approximately the same if one may assume similar sizes of their pools. This assumption seems reasonable, since the carbamyl carbon was incorporated to an equal extent into both purines and pyrimidines.

The authors would like to express their indebtedness to Dr. Norman Abrahamsen for the preparation of aminofumaric acid diamide-3-C^{14}, to Dr. Ezra Staple for the orotic acid-2-C^{14} and the L-citrulline-C^{14}-N^{15}, and to Dr. Sidney Weinhouse and the Houdry Process Company of Marcus Hook, Pennsylvania, for the N^{15} analyses.

SUMMARY

1. A method for the preparation of DL-dihydroorotic acid by the hydrogenation of orotic acid has been described.

2. Homogenates of rat liver are capable of converting ureidosuccinic acid into dihydroorotic acid and both of these compounds into orotic acid.

3. Citrulline appears not to be involved in pyrimidine formation in the rat.

4. Ureidosuccinic acid is converted to nucleic acid pyrimidines in slices of regenerating rat liver by way of orotic acid.

5. Aminofumaric acid diamide is a precursor of nucleic acid pyrimidines.

6. Some evidence of the structure of aminofumaric acid diamide is given. The ultraviolet absorption spectra were determined in neutral, acidic, and alkaline solutions.

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made of the amount of injected citrulline which was converted to urea and therefore, presumably, to argininosuccinic acid. If the latter compound were converted to both pyrimidines and urea, a low estimate of 1 per cent might be made for the amount which would be available for pyrimidines (7). Since 44 mg. of pyrimidines were recovered, it may be approximated that the specific activity of the pyrimidines was only about 1 per cent of that which would have been obtained had the urea cycle been involved in pyrimidine formation.

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METABOLISM OF SERINE AND GLYCINE PEPTIDES BY MUTANTS OF ESCHERICHIA COLI STRAIN K-12*

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In recent years, there have been a number of reports that peptides exhibit greater growth-promoting activity than free amino acids for both gram-negative and gram-positive organisms (1–13). Among the auxotrophic mutants of *Escherichia coli* strain K-12, this phenomenon has been found only with a prolineless mutant (strain No. 679-183), which grows better on a variety of synthetic proline peptides than it does on proline (4). Subsequent studies (8, 11) demonstrated that the superior activity of the peptides was due to the "protection" of proline bound in peptide linkage from degradation by the bacterial enzyme system acting on free proline. In the course of an investigation of the growth response of two serine-requiring mutants of *E. coli* strain K-12 to serine peptides, another example of the greatly enhanced activity of a synthetic peptide (L-serylglycine) has been observed.

Of the two auxotrophic mutants[1] used in the current study, strain S grows only in the presence of L-serine, but strain S/G can grow either on L-serine or on glycine (14). Strain S/G, therefore, also served as a test organism for an investigation of the bacterial utilization of glycine supplied in the form of glycine peptides. The latter series of experiments was of interest in view of the data already available concerning the growth response to glycine peptides of several auxotrophs of *E. coli* strain K-12 that have specific requirements for amino acids other than glycine (4, 8, 11, 15–20).

EXPERIMENTAL

The metabolism of L-serine (Ser) and glycine (Gly) by strains S and S/G has been described previously (14). As in the earlier experiments

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[1] The auxotrophic mutants of *E. coli* strain K-12 were made available through the courtesy of Dr. Joshua Lederberg of the Department of Genetics, University of Wisconsin.

with these amino acids (14), the growth tests with the peptides[2] were carried out in a final volume of 7 ml. of medium containing "small inocula" (about 2×10^6 cells) or "large inocula" (about 4×10^7 cells). All cultures were incubated on a shaker at 35°, and growth was followed as a function of time by periodic absorbancy (optical density) measurements made in an Evelyn colorimeter (filter No. 540). The efficacy of the peptides was compared to that of the free amino acids by determining, for each test compound, the "lag time" and the "generation time" of the growth cycle and the concentration, in millimoles per liter, required to produce maximal growth equivalent to an optical density of 0.15 (14).

Metabolism of Serine Peptides by Strains S and S/G

The growth response of the two auxotrophs to serine peptides and to mixtures of the constituent amino acids is summarized in Table I. As may be seen from the data in Table I, the concentration of a peptide required for growth equivalent to a maximal optical density of 0.15 (*i.e.* the "extent of growth") is markedly influenced by the number of cells in the inoculum. This is likewise true of the extent of growth on serine alone or supplemented with other amino acids.

L-*Seryl*-L-*serine* (*SerSer*)—With small inocula of both strains, the extent of growth on SerSer is only slightly greater than growth on free Ser, if it is assumed that both residues in the dipeptide are used by the organisms. Large inocula, however, do not grow in a medium in which SerSer is the sole exogenous source of Ser. On the other hand, when a large inoculum is provided with a mixture of Ser and SerSer, the growth is somewhat better than that expected on the basis of the free Ser supplied in the medium.

These observations may be explained by the assumption that the utilization of SerSer for growth requires the initial hydrolysis of the dipeptide and that the Ser liberated can be deaminated by the cells. Thus, in the presence of a large inoculum, the initial concentration of the requisite peptidase and serine deaminase would be so high that any free Ser is deaminated before it can be used for cell multiplication; with a small inoculum, hydrolysis of the peptide and destruction of Ser would not be sufficient to lower the Ser concentration to a level incapable of supporting growth. Several lines of evidence may be cited in support of this hypothesis. First, it is known that resting cells of strains S and S/G readily deaminate Ser (14). Secondly, under the conditions of the growth tests, the cells comprising a large inoculum remove all the SerSer from the medium without undergoing any detectable growth. This fact was es-

[2] The authors are greatly indebted to Dr. Joseph S. Fruton for the peptides that were used in this study.

tablished by preparing sterile filtrates from cultures of large inocula (strain S or S/G) incubated for 25 hours in the basal medium supplemented with

TABLE I

Growth Response to Serine Peptides

Each peptide was tested over the concentration range 0.1 to 1.0 mM and, unless noted otherwise, the concentration of the test compound permitting maximal growth equivalent to an optical density of 0.15 (concentration) was estimated from the concentration curve (14) constructed on the basis of measurements made after 24 hours incubation. The generation time during logarithmic growth was estimated to the nearest half-hour. The values given represent the average of two or more independent experiments in which the peptide was compared either to serine or to an equimolar mixture of its constituent amino acids.

Test compound	Strain S				Strain S/G			
	Small inoculum		Large inoculum		Small inoculum		Large inoculum	
	Concentration	Generation time	Concentration	Generation time	Concentration	Generation time	Concentration	Generation time
	mM	*hrs.*	*mM*	*hrs.*	*mM*	*hrs.*	*mM*	*hrs.*
L-Serine	0.43	1.5	0.75	2.0	0.46	2.0	0.75	2.5
L-Seryl-L-serine	0.18	1.5	No growth		0.18	2.0	No growth	
L-Serine + L-alanine	0.43 of each	2.5	0.75 of each	2.0	0.51 of each	2.0	0.84 of each	3 (*Ca.*)
L-Alanyl-L-serine	0.60*	2.0	No growth		0.56*	2.5	No growth	
L-Seryl-L-alanine	0.60*	2.5	" "		0.47*	2.5	" "	
L-Serine + glycine	0.32 of each	1.5	0.57 of each	1.5	0.21 of each	2.0	0.34 of each	2.0
Glycyl-L-serine			>0.7	3.0			0.73	2.5
L-Serylglycine	0.17	1.5	0.28	1.5	0.10†	1.5	0.27	2.0

* Values estimated from measurements made after 63 hours incubation due to the prolonged lag periods of these growth cycles. Under conditions whereby the lag time for serine and for serylserine is about 10 hours, that for alanylserine is 15 to 21 hours, and that for serylalanine is 12 to 16 hours.

† Tested in one experiment only.

SerSer (0.2 mM) and by testing the filtrates for their ability to support the growth of small inocula. Such filtrates did not contain any growth-promoting compounds; nor did they contain any substances that inhibit the

growth of small inocula in the presence of added Ser. Consequently, the filtrates must have been devoid of both SerSer and Ser.

Furthermore, during the lag period of the growth cycle of small inocula on SerSer, some of the dipeptide apparently is hydrolyzed, as shown by the following experiment. At the end of the lag period, cultures of small inocula (strain S or S/G) in media containing SerSer (0.2 mM) were centrifuged (to provide "centrifuged cells"), and the supernatant fluid was passed through a bacterial filter. When a small inoculum of strain S was added to a portion of the sterile filtrate, the growth of the culture was such as to indicate the presence of either SerSer at a concentration of about 0.1 mM, Ser at a concentration of about 0.2 mM, or an equivalent mixture of the dipeptide and the free amino acid. Since the sterile filtrate also supported the growth of a large inoculum of strain S, it may be inferred that free Ser was present. However, some SerSer must also have been present, for the extent of growth of the large inoculum was much less than would have been expected if the filtrate had been 0.2 mM with respect to Ser. Thus, approximately half of the SerSer to which the centrifuged cells were initially exposed could be accounted for (as SerSer plus Ser) by the bioassays on the sterile filtrate. The remainder of the SerSer apparently was taken up by the centrifuged cells, for, when these cells were resuspended in the sterile basal medium, the resultant culture grew to a maximal optical density characteristic of a small inoculum in a medium that is about 0.1 mM with respect to SerSer.

The growth of the centrifuged cells on resuspension in the basal medium illustrates the ability of these organisms to "concentrate" an essential growth factor. Only one other case of this kind has been encountered among the amino acid auxotrophs of *E. coli* strain K-12, that of the proline auxotroph (8), although the phenomenon is characteristic of several gram-positive bacteria (21).

Peptides of L-*Alanine and* L-*Serine*—L-Alanyl-L-serine (AlaSer) and L-seryl-L-alanine (SerAla) support only the growth of small inocula of strains S and S/G. The failure of large inocula to grow on AlaSer or SerAla is probably due to the factors discussed above in connection with the analogous results for SerSer. However, as may be seen from the data cited in Table I, the Ser residues of AlaSer and SerAla are used less efficiently than Ser provided as SerSer. It is of interest that the prolonged lag period on AlaSer (Table I) may be reduced by using an inoculum harvested from the basal medium supplemented with this dipeptide. This adaptive response, which is seen only with AlaSer, suggests that, if hydrolysis of AlaSer and SerAla is a prerequisite for the utilization of the Ser residues for growth, the two dipeptides are not cleaved by the same peptidase (22).

Although both strains show extended lag periods in the presence of AlaSer and SerAla, only strain S/G is inhibited by mixtures of L-alanine (Ala) and Ser. Thus, the addition of Ala to the medium does not alter significantly the growth response of strain S to Ser, but the growth cycles of strain S/G in media containing Ala and Ser usually have extended lag periods and never attain sharply defined growth maxima. Consequently, it is difficult to estimate accurately, for strain S/G, the relative growth-

TABLE II

Growth Response of Strain S/G to L-*Serine and to Equimolar Mixtures of* L-*Serine and* L-*Alanine*

The data from four representative experiments are given. All growth tests were carried out with small inocula.

Lag time*		Concentration of each amino acid inducing growth equivalent to optical density of 0.15 at various times after inoculation				
Serine	Serine + alanine	Serine†	Serine + alanine‡			
		22 hrs.	22 hrs.	24 hrs.	30 hrs.	40 hrs.
hrs.	*hrs.*	*mM*	*mM*	*mM*	*mM*	*mM*
10–11	11	0.46	0.54			0.35
11	13	0.46	0.62	0.58	0.52	0.46
12	14	0.46	0.63	0.54	0.44	0.35
10–11	17	0.46	1.0	0.54	0.49	0.38

* The duration of the lag period in each experiment was estimated from the curves describing the growth in media that contained L-serine (alone or supplemented with an equimolar amount of L-alanine) at concentrations of 0.3, 0.6, and 1.0 mM.

† All cultures grown on unsupplemented L-serine had attained their maximal optical density by 22 hours. The growth curves for these cultures have sharply defined maxima.

‡ In contrast to the growth curves for serine, the curves for the mixtures show a very long phase of "negative growth acceleration" following the logarithmic phase.

promoting property of the mixture compared to that of unsupplemented Ser (Table II).

An antagonism between Ala and Ser has been observed for several microorganisms (10, 23–25). In the case of *Lactobacillus delbrueckii*, which most closely resembles the situation obtaining in strain S/G, it has been suggested that Ala interferes with the uptake of Ser by the bacteria (10). That this explanation probably does not apply to the effect observed with strain S/G may be inferred from the fact that Ala does not inhibit the growth of strain S which, presumably, handles exogenous Ser in exactly the same way as strain S/G. The difference in the response of the two

E. coli mutants to mixtures of Ala and Ser also may be taken as evidence that the prolongation of the lag periods of the growth cycles of both strains by AlaSer and SerAla is not due to the action of Ala liberated by hydrolysis of the dipeptides but reflects the necessity for the production of the bacterial enzyme systems that act on these compounds.

Peptides of Glycine and L-*Serine*—The failure of glycyl-L-serine (GlySer) to support the growth of either strain S or strain S/G to an extent comparable to growth on a mixture of its component amino acids (Table I) suggests that some of the Ser liberated by the hydrolysis of the dipeptide is immediately deaminated. However, the ability of large inocula of the two mutants to grow in the presence of GlySer shows clearly that the Ser residue of this dipeptide is protected from the degradative reactions which make the Ser residues of AlaSer, SerAla, and SerSer unavailable for the purposes of cell multiplication.

L-Serylglycine (SerGly) is much more efficient than either GlySer or the equivalent mixture of free amino acids in supporting the growth of both small and large inocula of the two mutants; this is especially striking in the case of strain S (Table I). Because the data for SerSer and SerAla indicated that the presence of a Ser residue as the amino end-group in a dipeptide does not protect Ser from the catabolic action of large inocula, it was difficult to explain the excellent growth-promoting properties of SerGly in such terms. Attention was therefore directed toward exploring the possibility that the utilization of SerGly for growth may not involve prior hydrolysis, and an attempt was made to determine whether strain S is capable of effecting such a hydrolysis and whether the mutant differed from its parent wild type, strain K-12, in this respect.

For use in the enzyme studies, extracts were prepared from strain S grown in the basal medium containing Ser (1 mM) and from strain K-12 grown in the unsupplemented basal medium. Cultures of each strain were incubated under forced aeration at 35° for 24 hours, the cells were collected by centrifugation and washed with cold 0.9 per cent saline solution, and the washed cells were ground for 15 to 30 minutes with alundum (60 mesh). After the addition of a small volume of saline, the mixture was centrifuged and the supernatant fluid (cell extract) was tested immediately for its peptidase activity toward SerGly. The extent of hydrolysis of the dipeptide was determined by the method of Schwartz and Engel (26), which involves an initial precipitation of protein with picric acid, followed by the photometric determination of amino nitrogen by the ninhydrin method of Moore and Stein (27). The course of the hydrolysis also was followed by paper chromatography with pyridine-water (65:35) as the developing solvent.

The data obtained in one experiment with an extract of strain S are

given in Table III, which shows that the initial disappearance of the dipeptide is accompanied by the appearance of both of its component amino

TABLE III

Hydrolysis of L-*Serylglycine by Extract of Strain S*

The enzyme experiments were carried out at 38° in a final volume of 2 ml. of a solution containing inorganic salts and phosphate buffer (pH 6.7). This solution was identical, except for the omission of glucose and all nitrogen compounds, to the basal medium used in the growth tests (14). Also present in the incubation mixture were L-serylglycine (0.01 M), L-cysteine (0.005 M), manganous sulfate (0.001 M), and 0, 0.2, 0.4, or 0.6 ml. samples of the cell extract. The nitrogen content of the cell extract was determined by micro-Kjeldahl analysis. When samples were removed for determination of the extent of hydrolysis by the method of Schwartz and Engel, approximately 0.005 ml. of each sample was analyzed by paper chromatography. In pyridine-water (65:35) as the developing solvent, the R_F values were L-serylglycine (SerGly) 0.69 (yellow spot that changes to purple in 24 to 48 hours), L-serine (Ser) 0.61, and glycine 0.51. The symbol ± is used to designate a weakly positive ninhydrin spot.

Incubation time	0.25 mg. bacterial N in cell extract			0.50 mg. bacterial N in cell extract			0.75 mg. bacterial N in cell extract		
	Hydrolysis	Chromatogram*		Hydrolysis	Chromatogram*		Hydrolysis	Chromatogram*	
		SerGly	Ser		SerGly	Ser		SerGly	Ser
hrs.	*per cent*			*per cent*			*per cent*		
2.5	15	+	+	16	+	+	17	+	+
5	29	+	+	26	+	+	17	+	+
10	34	+	+	50	+	+	51	+	+
23	52	+	±	66	−	±	62	−	±
26	52	±	±	65	−	±	63	−	±
29	53	−	±	62	−	±	64	−	±
34	71†	−	−	84†	−	−	84†	−	−

* At 0 hour, only SerGly was detected. Chromatograms of all subsequent samples showed the presence, in the incubation mixture, of glycine as well as the compounds indicated in the table.

† Calculated on the assumption that all the ninhydrin color in the amino nitrogen determination was due to glycine. Independent experiments showed that resting cells of strain S begin to deaminate glycine at a rapid rate only after exposure to the amino acid for about 24 hours (14).

acids. However, at the end of the incubation period, only Gly is detectable on the chromatograms. Similar results were obtained in analogous experiments with extracts of strain K-12. It is not possible, however, to draw any conclusions concerning the relative peptidase activity of the two strains because the analytical data calculated by the method of Schwartz and Engel are, at best, only minimal values.

TABLE IV

Growth Response of Strain S/G to Glycine Peptides

Unless otherwise stated, each peptide was tested over the concentration range 0.1 to 1.0 mM, and the values given represent the average of two or more independent experiments in which the peptide was compared either to glycine or to an equimolar mixture of its constituent amino acids. The concentration permitting maximal growth equivalent to an optical density of 0.15 (concentration) was estimated from the density measurements representing the maximal growth of the cultures regardless of the time such growth was attained. All peptides containing proline were prepared in aqueous solutions, which were sterilized by filtration and added to the autoclaved basal medium.

Test compound	Small inoculum		Large inoculum	
	Concentration	Generation time	Concentration	Generation time
	mM	*hrs.*	*mM*	*hrs.*
Glycine	0.37	2.5	0.37	2.0
Glycylglycine	0.16	4–8*		
Glycine + L-tryptophan	0.27 of each	2.0	0.22 of each	2.5
Glycyl-L-tryptophan	0.47	11.0†	0.22‡	6.0
Glycine + L-phenylalanine	0.35 of each	3.5		
Glycyl-L-phenylalanine	0.46	5.0	0.44§	1.5
L-Phenylalanylglycine	0.49‡	2.5		
Glycyldehydrophenylalanine	No growth		No growth	
Glycine + L-tyrosine	0.35 of each	3.5		
Glycyl-L-tyrosine	0.40	3.5	0.40‡	1.5
L-Tyrosylglycine	0.38‡	5.0		
Glycine + D-leucine	0.33 of each‡	2.0	0.43 of each‡	1.5
Glycyl-D-leucine	No growth		No growth	
D-Leucylglycine	" "		" "	
Glycine + L-leucine	0.33 of each	2.0	0.40 of each	2.5
Glycyl-L-leucine	0.35	3.5	0.73	5.5
L-Leucylglycine	0.35	4.0	0.73	5.5
Glycine + L-alanine	0.39 of each	2.5	0.39 of each	2.0
Glycyl-DL-alanine	0.64‡			
L-Alanylglycine	0.29‡			
Glycine + L-proline	0.35 of each	2.5		
Glycyl-L-proline	0.3 (*Ca.*)§	6.0	0.25 (*Ca.*)§	2.0
L-Prolylglycine	0.75 (*Ca.*)§		0.5 (*Ca.*)§	13.0
Glycyl-L-prolylglycine	0.24‡	11.0	0.16‡	4–11.5‖
Diglycylhydroxy-L-proline	0.35‡	11.0 (*Ca.*)‖	0.17‡	

* The generation time increases as the initial concentration of glycylglycine in the medium is increased.

† The lag time of the growth cycle was 35 hours. On reinoculation into a medium containing glycyltryptophan, the lag time of the resultant cultures was 20 hours and the generation time 4 hours.

TABLE IV—*Concluded*

‡ Tested in only one experiment.

§ Estimated from the activity of the peptide at a single concentration (0.6 mM) compared to that of an equivalent concentration of glycine.

‖ The generation time decreases as the initial concentration of the tripeptide in the medium is increased.

In view of the finding that cell extracts of strain S hydrolyze SerGly and destroy the Ser liberated, the growth response of the auxotroph to the dipeptide was reinvestigated in an attempt to learn whether similar reactions occur during the growth cycle. For these experiments, strain S (both large and small inocula) was cultured in a medium containing a mixture of Ser (0.5 mM) and Gly (0.5 mM) or SerGly (0.5 mM). At intervals, 0.1 ml. samples were removed aseptically from each culture and subjected to chromatographic analysis as described above. After 24 hours incubation, sterile filtrates were prepared from each culture and were assayed microbiologically for Gly with strain S/G as the assay organism.

From the chromatograms of all media initially supplemented with the mixture of Ser and Gly, it was apparent that Ser gradually disappears from the culture fluid during the growth cycle, the time at which this disappearance occurs being identical with the logarithmic phase of growth. Gly is always present in these media, and, according to the results of the bioassay, 80 to 100 per cent of the free Gly initially present in the media is still there at the end of the incubation period. Chromatograms of media initially supplemented with SerGly show that some free Gly and Ser appear in the culture fluid during the lag phase, and that the Ser, as well as the dipeptide, disappears from the media during logarithmic growth. The bioassays of filtrates prepared at the end of growth on the dipeptide indicate the presence of 1 to 2 per cent of the Gly initially supplied as SerGly. It may be inferred, therefore, that the extracellular hydrolysis of SerGly does not occur to any significant extent, and, if intracellular hydrolysis occurs, very little of the Gly formed is secreted by the cells into the medium. Although these data do not permit any conclusion regarding the occurrence of the hydrolysis of SerGly as the first step in the utilization of the peptide Ser for growth, they show clearly that the response of strain S to both Ser and Gly supplied as free amino acids differs significantly from the response of this mutant to the Ser and Gly residues of the dipeptide.

Metabolism of Glycine Peptides by Strain S/G

The growth response of strain S/G to a number of Gly peptides is summarized by the data in Table IV; the utilization of GlySer and SerGly by this strain has been discussed above. As may be seen from Table IV, the Gly residues of most of the peptides were ultimately utilized for growth, but the rate of growth on the compounds tested varied widely. In several

cases, the lag times also differed from that characteristic of growth on Gly, which was 12 to 15 hours for small inocula and 4 to 6 hours for large inocula (14).

Glycylglycine (*GlyGly*)—Unlike the growth response of strain S/G to any other glycine peptide tested, the rate of growth on GlyGly decreases as the initial concentration of the peptide in the medium is increased. Since the growth rate on free Gly is independent of the concentration used, and since all the potential Gly supplied as the dipeptide is ultimately utilized for growth, the inhibitory effect of GlyGly on the growth rate probably is overcome by cleavage to yield Gly. When GlyGly was tested on two serine-glycine auxotrophs of *E. coli* strain W (14), the concentration of the dipeptide in the medium did not influence the rate of growth of either mutant.

Glycyl-L-tryptophan (*GlyTry*)—With small inocula of strain S/G, the lag time of the growth cycle on GlyTry is very long, the growth rate is extremely slow, and the extent of growth is small compared to growth on Gly or on a mixture of Gly and L-tryptophan (Try). The last result may be due to the fact that some of the Gly formed by the slow hydrolysis of the peptide during the prolonged lag period is deaminated (14) before the concentration of free Gly reaches a level capable of supporting cell multiplication. The utilization of GlyTry by small inocula appears to be the result of an adaptation, since a subculture of cells grown on the peptide utilizes it much more readily than do cells harvested from agar slants containing peptone and yeast extract. The relative speed with which a large inoculum grows on GlyTry suggests that the unadapted cells comprising a large inoculum merely provide a relatively high initial concentration of the adaptive enzymes in the medium.

Equimolar mixtures of Gly and Try permit more extensive growth of both small and large inocula than that obtained with unsupplemented glycine. The reason for this is obscure; Try has no effect on the growth response of strain S/G to Ser, and, if the aromatic amino acid "spares" the requirement for Gly, it should likewise spare the requirement for Ser.

Peptides of Glycine and L-Phenylalanine or L-Tyrosine—The extent of growth on glycyl-L-phenylalanine (GlyPhe) and on L-phenylalanylglycine (PheGly) is equal, but the latter permits faster growth of small inocula. Presumably, the rate of hydrolysis of GlyPhe is so slow that it becomes the limiting factor controlling the rate of growth. This conclusion is supported by the observation that, with a large inoculum (*i.e.* a higher initial concentration of the appropriate peptidase), the growth rate on the dipeptide is markedly increased. In this connection, it may be noted that a phenylalanine auxotroph (No. 58-278) of strain K-12 grows as rapidly on GlyPhe as it grows on L-phenylalanine (Phe) (15, 18). However, the phenyl-

alanine auxotroph requires Phe at a concentration of about 0.01 mM for a maximal optical density of 0.15, and a slow hydrolysis of the dipeptide might still permit the release of sufficient free Phe to support growth at a normal rate. Neither the phenylalanine auxotroph nor strain S/G grows on glycyldehydrophenylalanine, and the inability of the former to use the peptide probably reflects the inability of both these mutants to hydrolyze the peptide to Gly and phenylpyruvic acid, since the keto acid has growth factor activity for strain 58-278 (15).

In the case of the dipeptides containing tyrosine (Tyr), glycyltyrosine permits faster growth than tyrosylglycine. Although, as suggested on the basis of experiments with the phenylalanine auxotroph and with a tyrosine auxotroph (No. 58-5030), analogous Phe and Tyr peptides may be cleaved by the same bacterial peptidases (15, 28), the introduction of the phenolic hydroxyl group into the Phe residue apparently exerts different effects on the activity of the enzymes in strain S/G that act on dipeptides in which the aromatic amino acid is present as the amino and the carboxyl end-group, respectively.

Peptides of Glycine and Leucine or Alanine—Strain S/G and a leucine auxotroph (No. 679-680) of strain K-12 are similar in their inability to grow on peptides of D-leucine (20). Strain S/G also lacks the ability to derive Gly from glycyl-D-alanine (supplied in the medium as glycyl-DL-alanine). It may be inferred, therefore, that enzymes acting on peptides of D-amino acids do not occur in the auxotrophs derived from strain K-12. This conclusion is supported by the fact that extracts of a methionine auxotroph (No. 58-181), which is the only mutant of strain K-12 known to utilize the D enantiomorph of its required amino acid, were found to be incapable of hydrolyzing D-methionylglycine and glycyl-D-methionine (19).

Strain S/G grows equally well on glycyl-L-leucine and L-leucylglycine, but large inocula do not grow to the expected extent on these two peptides. The growth curves of both small and large inocula on each peptide are characterized by lag times that increase as the initial concentration of the test compound is raised. This type of bacteriostatic action by L-leucine peptides was encountered previously with the leucine auxotroph, a threonine auxotroph (No. 679), and strain K-12 (20). Furthermore, at concentrations comparable to those required to produce growth of small inocula of strain S/G equivalent to an optical density of 0.15 (Table IV), the peptides also caused an increase in the generation time and a decrease in the extent of growth of the leucine auxotroph (20). The earlier suggestions that the inhibitory peptides block the incorporation of leucine into peptide linkage and that the bacteriostasis is overcome when the inhibitory peptide is cleaved by the bacterial peptidases (20) apply equally well to the data obtained with strain S/G.

Peptides of Glycine and L-*Proline*—At the one concentration tested (0.6 mM), glycyl-L-proline (GlyPro) was utilized much better than L-prolylglycine (ProGly). This finding is in agreement with the data obtained in a comparison of the metabolism of the two peptides by the proline auxotroph of strain K-12 (4, 8, 11). All the proline supplied as ProGly can ultimately be utilized for growth by the proline auxotroph under conditions in which the bacterial deamination of proline is reduced to a minimum, but strain S/G does not use all the Gly potentially available in the dipeptide. Only large inocula of strain S/G use all the Gly supplied as glycyl-L-prolylglycine (GlyProGly), but even small inocula derive more Gly from the tripeptide than they do from ProGly. On the basis of the relative growth-promoting activities of the GlyProGly, GlyPro, and ProGly, it may be inferred that the utilization of GlyProGly for growth involves an initial slow cleavage of the tripeptide to GlyPro, followed by a more rapid hydrolysis of GlyPro. The data from growth tests with the proline auxotroph may be interpreted in the same way, and this mutant has been shown to be capable of cleaving both peptide bonds of GlyProGly (11). The growth response of strain S/G to GlyProGly represents a case in which the rate of growth is increased when the initial concentration of peptide in the medium is raised. A similar effect is seen with the tripeptide glycylglycylhydroxy-L-proline. For both tripeptides the lag period is abnormally long, and the growth response to these compounds may require an adaptive period during which the requisite "tripeptidases" are formed.

Other Glycine Peptides—In addition to the peptides discussed above, glutathione also was tested and was found to have no growth-promoting activity for strain S/G. Glutathione likewise is inactive as a growth factor for the cystine auxotroph (No. 58-309) of strain K-12.[3] It is not known, however, whether this tripeptide is taken up by the bacteria under the conditions of the growth tests.

DISCUSSION

The results obtained both with Ser peptides and with Gly peptides are consistent with the hypothesis that the metabolism of these compounds in growing cultures of amino acid auxotrophs of *E. coli* strain K-12 follows the same general pathway in which the peptides are hydrolyzed before the "essential" amino acid can be used for the catabolic reactions associated with cell multiplication.

None of the Gly peptides (other than SerGly) is significantly superior to a mixture of its constituent amino acids in supporting the growth of strain S/G, and some of the peptides are used less well than the corresponding amino acid mixtures. The latter phenomenon is especially striking if

[3] Simmonds, S., and Radford, M., unpublished data.

"use" is described in terms of the rate rather than the extent of growth. The observed variations in the growth rates probably reflect the fact that strain S/G requires such a high initial concentration of Gly for growth that, if the rate at which a peptide is hydrolyzed is slow, the concentration of free Gly at any given time may be too low to support growth at a normal rate.

The data comparing the utilization of Gly peptides by strain S/G and by the phenylalanine, tyrosine, leucine, and proline auxotrophs indicate that the bacterial growth response is much the same, whether Gly or another amino acid residue provides the factor required for growth. The differences in the rate and extent of growth of two mutants on the same peptide in most cases may be attributed to known metabolic reactions. Thus, as noted above, the slow hydrolysis of a peptide may limit the growth rate of a glycine-requiring auxotroph like strain S/G, but it need not limit the growth rate of another amino acid auxotroph having a much lower requirement for its essential amino acid. Likewise, the failure of strain S/G to utilize all the Gly supplied in a given peptide, and the full utilization of the other amino acid residue by the appropriate mutant, may be a result of the bacterial deamination of the Gly formed by hydrolysis of the peptide. Such deamination of Gly probably is of significance only in a culture that has a long lag period and a slow rate of growth, since resting cells of strain S/G do not readily deaminate Gly during the first 15 to 20 hours of incubation in the presence of the amino acid (14).

Because strains S and S/G deaminate Ser very rapidly (14), this catabolic reaction should play an important rôle in determining the growth response to Ser peptides. The observed differences in the ability of Ser peptides to serve as growth factors suggest that, if the rate at which Ser is liberated from a peptide during the lag phase of growth is slow, the subsequent deamination of Ser may be so rapid that the concentration of Ser never becomes sufficiently high to support growth. The fate of the Ser residue of a peptide, therefore, is markedly influenced by the relative activities of the bacterial enzyme systems that catalyze the hydrolysis of the peptide, the deamination of Ser, and the reactions associated with the utilization of Ser for cell growth and multiplication.

It has been pointed out previously (21) that the bacterial utilization of a peptide amino acid for growth may follow pathways other than that involving the initial hydrolysis of the peptide. That an alternative reaction sequence is operative in the growth response to SerGly is strongly indicated, for, if the Ser residue of that dipeptide is used better than free Ser because it is protected from the catabolic activity of the organisms, then the serine residue of SerAla (if not of AlaSer, GlySer, and SerSer) should likewise be protected, and it is not. However, the possibility cannot be overlooked

that the hydrolysis of SerGly, unlike that of SerAla, occurs much more rapidly than the subsequent deamination of Ser. If any of the exogenous SerGly is used for growth without prior hydrolysis, it may participate in transpeptidation reactions leading to the incorporation of Ser into the bacterial proteins. The ability of crude extracts of strain S to catalyze the hydrolysis of SerGly is not incompatible with this hypothesis, since many enzymes responsible for the hydrolysis of peptides have been shown to catalyze transpeptidation reactions (29). Still another metabolic pathway that might be followed is the direct incorporation of SerGly into proteins. Relevant to this idea are the reports that DL- and L-serylglycyl-L-glutamic acids have "strepogenin" activity for several lactobacilli (2, 3).[4]

The experiments with strain S grown on SerGly or on the mixture of Ser and Gly also illustrate the significant difference that may exist in the bacterial metabolism of a "dispensable" amino acid (*i.e.* Gly) when it is supplied in the medium as the free amino acid or as a peptide. Such a difference in the fate of free Gly and peptide Gly has been observed not only in growth tests with strain S, but also in growth tests and deamination experiments with the proline auxotroph (4, 8, 11). Of special interest in this connection is the observation (30) that growing cultures of strain K-12 (as well as of the proline auxotroph) incorporate less N^{15} into their proteins when they are supplied with glycine-N^{15} and unlabeled L-proline than when they are given glycyl-N^{15}-L-proline; an enhanced incorporation of N^{15} also results when a mixture of either glycyl-N^{15}-glycine or glycyl-glycine-N^{15} plus unlabeled proline is supplied in the medium.

SUMMARY

The growth response of two serine-requiring mutants (strains S and S/G) of *E. coli* strain K-12 to several serine dipeptides is consistent with the view that the utilization of peptide serine for growth involves the prior hydrolysis of the peptide bond. The serine so liberated may be used in the anabolic reactions associated with growth or may be deaminated by the bacteria prior to the initiation of growth.

L-Serylglycine proved to be a better growth factor than L-serine alone or supplemented with glycine. This peptide was hydrolyzed and the serine degraded by extracts of strain S, but its superior growth-promoting action cannot be accounted for solely on the basis of "protection" of the serine residue from the catabolic reactions to which free serine is subjected.

No glycine peptide (other than serylglycine) was superior to a mixture

[4] Preliminary experiments in which L-serylglycyl-L-glutamic acid (0.6 mM) was tested as a growth factor for strain S indicate that the tripeptide is only one-tenth as active as an equivalent mixture of L-serine and glycine (alone or supplemented with L-glutamic acid).

of its constituent amino acids as a growth factor for strain S/G. The growth response of strain S/G to the glycine peptides was qualitatively similar to the response of other amino acid auxotrophs for which the non-glycine residue of the peptides provided the required growth factor. Hydrolysis of the glycine peptides apparently is a prerequisite to the utilization of the amino acid residue required for growth by each of the auxotrophs studied.

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PURIFICATION AND PROPERTIES OF D-GLUCOSE-6-PHOSPHATE DEHYDROGENASE*

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D-Glucose-6-phosphate dehydrogenase (*Zwischenferment*) was discovered by Warburg and Christian in 1931 (1, 2). An active preparation was obtained then from horse erythrocytes and in 1932 from the Lebedev juice made from brewers' yeast (3). Because of its extreme specificity, this enzyme has become an important analytical tool. Various preparations of it have been described, the first being those of Warburg and Christian (1–5) who found that the active protein could be precipitated from diluted Lebedev juice by acidification with carbon dioxide. We have been able to effect a 5- to 8-fold purification of the dehydrogenase by this method, but the conditions are not reproducible from one lot of yeast to another. Negelein and Gerischer (6) were able to obtain a preparation about 65-fold purified, in small yield, after an intricate fractionation procedure. More recently Kornberg has described a duplicable procedure which yields a product purified 20-fold and in good yield (7). In this paper we report the further purification of the enzyme and some of its properties.

EXPERIMENTAL

Materials and Methods—The pure crystalline barium salt of D-glucose-6-phosphate (G-6-P) and triphosphopyridine nucleotide (TPN) of 72 per cent purity was obtained from the Sigma Chemical Company. The δ-lactone of gluconic acid was a commercial sample from General Biochemicals, Inc. Ribose nucleic acid (RNA) and protamine sulfate were purchased from the Nutritional Biochemicals Corporation, while ethylenediaminetetraacetic acid (EDTA) was a product, recrystallized three times, from the Alrose Chemical Company. Adenosine triphosphate (ATP) was purchased from the Pabst Laboratories.

Calcium phosphate gel was prepared by the method of Keilin and Hartree (8) and alumina C_γ by a slight modification of the method of Willstätter (9). The former gel had 16 mg., while the latter had 19 mg. dry weight of solids per ml.

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Reduced TPN (TPNH) was prepared by treatment of the nucleotide with $Na_2S_2O_4$, followed by precipitation with acetone (10).

Dried brewers' yeast was generously supplied by Anheuser-Busch, Inc., of St. Louis. The yeast was washed with cold water by decantation, following its removal from the fermentation vats, and then was cold-pressed and cold-dried in an air stream at 1–2°. The dry yeast was stored at 5°. Enzyme preparations of the same activity were obtained from this yeast over a period of 6 months.

Protein was determined by the method of Robinson and Hogden (11), or, occasionally, by the method of Lowry *et al.* (12). When the latter method was used, care was taken to wash the protein repeatedly with 5 per cent trichloroacetic acid to free it from any adhering ammonium sulfate. Crystalline bovine plasma albumin (Armour) of known nitrogen content (Kjeldahl) was used as a standard.

The assay for the enzyme was done in the 1 cm. cell of the model DU Beckman spectrophotometer. The routine assay system contained 1.6 ml. of water, 1.0 ml. of 0.19 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 0.10 ml. of 0.30 M $MgCl_2$, 0.10 ml. of 0.025 M dipotassium glucose-6-phosphate, pH 7.0, and 0.20 ml. of 0.002 M TPN, previously adjusted to pH 6.8 with KOH. 1 unit of enzyme is defined as that quantity added to the above assay system which produces an optical density change at 340 mμ of 2.07 units per minute per 3 ml. of reaction mixture (1.0 μmole of TPN reduced per minute) at 25°. Optical density readings were made at intervals of 15 seconds and the activity was determined from the initial linear rate.

Enzyme Preparation. Autolysis—Dry brewers' yeast (200 gm.) was ground to a fine powder in a mortar and then allowed to autolyze in 600 ml. of distilled water for 6 hours at 37°. Table I presents the analytical data on such an autolysate as well as on the fractions obtained from it as described below. Other preparations of brewers' yeast might require somewhat different conditions of autolysis for optimal yield of the dehydrogenase. The autolysate was centrifuged in the cold at 15,000 × *g* for 15 minutes. The opalescent supernatant fluid (350 to 380 ml.) was kept frozen overnight without change in activity. All operations hereafter were carried out between 0–4° unless otherwise stated.

Protamine Precipitation and Ammonium Sulfate Fractionation—The nucleic acid in the autolysate was precipitated by the addition of 0.5 its volume of 2 per cent protamine sulfate solution, removed by centrifugation at 15,000 × *g* for 15 minutes, and discarded. The supernatant fluid was fractionated by the addition of unneutralized ammonium sulfate saturated at 25°. The protein fraction which precipitated between 0.575 and

0.65 saturation[1] was collected by centrifugation and dissolved in 100 ml. of 0.001 M EDTA, pH 7. This solution could be kept frozen for several days.

Calcium Phosphate Gel Adsorption—The solution was dialyzed against two 3 liter portions of 0.001 M EDTA, pH 7, for a total time of 3 hours. Dialysis could not be continued beyond this time without loss of enzymatic activity. After removing any precipitate present, the activity of the dialyzed solution was determined. For every 100 units of enzyme present, 3.1 ml. of calcium phosphate gel were added and allowed to stand for 5

TABLE I

Purification of D-*Glucose-6-phosphate Dehydrogenase*

Fraction	Volume	Total activity	Specific activity	Over-all recovery
	ml.	*units*	*units per mg.*	*per cent*
Autolysate	350	1050	0.13	100
$(NH_4)_2SO_4$ fraction, 0.575–0.65 saturated	110	725	0.34	69
$Ca_3(PO_4)_2$ gel eluate	45	562	3.45	53
Ethanol (9–20% volume per volume)	13	245	10.0	23
Alumina C_γ eluate	10.5	220	16.7	21
Column Fraction* 1†	2	5.4		
" " 2	2	25.4	86.0	
" " 3	1.5	21.7	86.8	
" " 4	2	21.6	75.6	
" " 5	2	17.4	65.0	
" " 6	2	13.6	68.0	
" " 7	2	8.5		
Total (Fractions 1–7)	13.5‡	113.6‡		19‡

* 120 units of enzyme activity added to column.

† First 0.40 saturated $(NH_4)_2SO_4$ eluate.

‡ Total in Fractions 1 through 7.

minutes with frequent stirring before centrifugation at 1000 × *g*. Without washing the gel the enzyme was eluted with 6.2 ml. of 0.3 M potassium phosphate buffer, pH 8, for every 100 units of activity present originally. The eluate could be kept frozen for several months without loss of activity.

Ethyl Alcohol Fractionation—The eluate was dialyzed for 8 to 8½ hours against several 2500 ml. portions of a solution 0.02 M in $MgCl_2$ and 0.001 M in EDTA, pH 7.0. Excessive loss of enzyme occurred when dialysis was continued for a longer time. A shorter time was insufficient for the re-

[1] All concentrations of $(NH_4)_2SO_4$ and of ethanol described in this procedure were calculated on the assumption of additive volumes.

moval of enough phosphate ion to permit a successful ethanol fractionation. At the end of dialysis the solution was centrifuged, and the supernatant fluid at 0° was adjusted to pH 5.9 (Beckman pH meter set at 10°) by the addition of 0.1 N HCl. 1 ml. of 95 per cent ethanol (at −6°) was added to the enzyme solution at 0°, and the solution was transferred immediately to a bath at −6°. The solution was made 9 per cent in 95 per cent ethanol, and the precipitate was quickly removed by centrifugation for 2 minutes at 1000 × *g* by using tubes and cups precooled to −10°. To the faintly turbid supernatant fluid at −6° enough 95 per cent ethanol at −6° was added to make its final concentration 20 per cent. The precipitate containing the enzyme was removed by centrifugation as above for 5 minutes. The precipitate was dissolved immediately in 10 to 12 ml. of 0.02 M $MgCl_2$-0.001 M EDTA, pH 7. Occasionally, it was necessary to add enough $MgCl_2$ to make its final concentration 0.05 M to effect solution of most of the precipitate. Any undissolved protein was removed by centrifugation. The enzyme was not stable on standing in this solution, and the preparation was carried at once through the next step.

Alumina Cγ Adsorption—For every 100 units of enzyme present 0.86 ml. of alumina Cγ was added. After 5 minutes the gel was removed by centrifugation, and, without washing it, the enzyme was eluted with 4.3 ml. of 0.10 M potassium phosphate-0.001 M EDTA buffer, pH 8.0, for every 100 units of enzyme originally present. After removing the gel by centrifugation at low speed, 3.5 ml. of 0.25 M glycylglycine buffer, pH 7.2, were added to the eluate. The solution was lyophilized, the white powder remaining was dissolved in 3 ml. of cold distilled water, and the mixture was centrifuged. The supernatant solution was quite stable when frozen.

Chromatography on Starch-Celite—The enzyme was purified further by applying the type of chromatography used by Fischer and Hilpert (13). A starch-Celite column was prepared by mixing 18 gm. of corn-starch with 18 gm. of Celite and packing the mixture into a 2 cm. × 18 cm. tube. Air pressure equivalent to 16 cm. of mercury was used to facilitate this and subsequent operations. 1 day before use, the column was washed at 4° under pressure with 50 ml. of cold distilled water followed by 50 ml. of 0.50 saturated ammonium sulfate, prepared, as were all ammonium sulfate solutions for this column, by suitable dilution of the neutralized (with NH_4OH to pH 7) salt solution saturated at 4°.

The enzyme solution from the preceding step was thawed, and that volume which contained 120 units of activity was brought to 0.56 saturation with the cold, neutralized, saturated ammonium sulfate. The faint precipitate which formed was removed by centrifugation in the high speed head of the International centrifuge.

The supernatant solution was added to the column and an equal volume of fluid was displaced by air pressure. 30 ml. of 0.60 saturated ammonium

sulfate were added to the column and forced through at a flow rate of 0.5 ml. per minute. After all of this solution had passed through, it was replaced by a 0.40 saturated solution. As this latter eluent was forced through the column and collected in 2 ml. fractions, the enzyme was found to emerge with the front of the 0.40 saturated ammonium sulfate. In the eluates containing the enzyme the protein concentration was very low; nevertheless, the enzyme was stable and withstood repeated rapid freezing and thawing.

The activity of the best fractions from the starch column varies from as low as 70 units per mg. of protein to as high as 120 units per mg. of protein, corresponding to a turnover number of 12,000 moles of substrate per 10^5 gm. of protein per minute at pH 8.0 at 25°. Thus, the over-all procedure for D-glucose-6-phosphate dehydrogenase purification results in a product 600 to 900 times purified from the Lebedev juice. The final preparation is free of phosphoglucomutase, hexose-6-phosphate isomerase, and 6-phosphogluconic acid dehydrogenase. However, hexokinase is still present, the ratio of the activities being approximately 40:1 in a column fraction with specific activity of 80 units per mg. of protein. The hexokinase assay was made via the glucose-6-phosphate dehydrogenase system by determining the initial rate of glucose utilization in the presence of an excess of all reactants. A preparation in which the hexokinase has been removed until the ratio of activities is 130:1 can be obtained as described below.

Further Removal of Hexokinase—The preparation was carried as far as the ethanol fractionation described above. Instead of adsorbing the enzyme on alumina $C\gamma$, 2.0 ml. of 0.25 M glycylglycine buffer, pH 7.2, were added for every 5 ml. of enzyme solution. The solution was dried by lyophilization and the powder was dissolved in enough cold water to give a final concentration of 60 units per ml. To 0.4 ml. of this solution was added 1.0 ml. of 1 M potassium acetate buffer, pH 4.7, followed by dilution with water to 8.0 ml. After standing at 0° for 10 minutes, the enzyme was precipitated by the addition of 3.0 ml. of 0.3 per cent RNA, pH 4.8. After standing for 10 minutes in ice the precipitate was removed by centrifugation. Solution of this precipitate in 0.25 M glycylglycine buffer, pH 7.2, gave a stable preparation in which the D-glucose-6-phosphate dehydrogenase had a specific activity of 20 units per mg. of protein. The yield was 50 per cent, starting with the ethanol-fractionated product. It has been observed that precipitation of the enzyme-RNA complex does not occur in solutions with high phosphate ion concentration. We have been unable to remove the last traces of hexokinase by reprecipitation with RNA.

Properties of Enzyme—Fig. 1 illustrates the effect of pH on the activity of the enzyme under the conditions of the standard assay procedure. The maximal activity occurs at about pH 8.5. The data for Fig. 1 were ob-

tained with organic acids and bases and their potassium and hydrochloride salts. As shown in Fig. 1 the activity was independent of the buffer used at pH 6.8, 7.55, and 9.0.

The effect of temperature on enzyme activity was studied by allowing the reaction to proceed at pH 7.4 in the presence of hydroxylamine which reacts with the lactone of 6-phosphogluconic acid to form the corresponding hydroxamic acid (14, 15) which can be determined quantitatively (16).

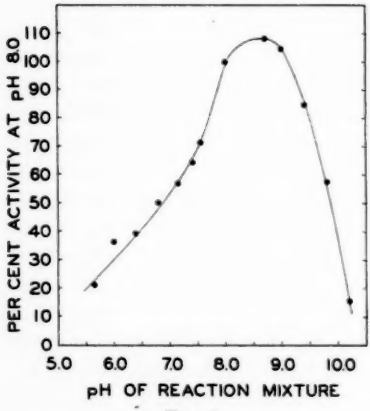

FIG. 1

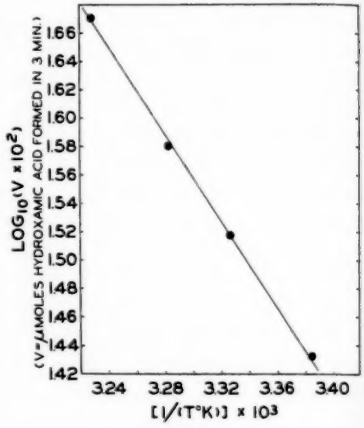

FIG. 2

FIG. 1. Variation of activity with pH. All incubations carried out in standard assay system with the following buffers: 0.19 M malonic acid, pH 5.6 to 6.8; 0.19 M maleic acid, pH 6.8 to 7.55; 0.19 M Tris, pH 7.55 to 9.0; 0.19 M glycine, pH 9.0 to 10.2. Enzyme (80 units per mg.) present at 0.5 γ per ml.

FIG. 2. Effect of temperature on enzymatic activity. The reaction mixtures (2.0 ml.) contained 9.5×10^{-2} M Tris, 2.5×10^{-3} M G-6-P, 5.0×10^{-4} M TPN, 1.5×10^{-2} M $MgCl_2$, 1.0×10^{-1} M KCl, 1.0×10^{-1} M NH_2OH; pH 7.40. Enzyme (15 units per mg.) present at 5 γ per ml. For the procedure, see the text.

Thus, when the enzymatic reaction was allowed to go to completion in the presence of hydroxylamine under conditions in which the amount of TPN was limiting, the color obtained in the hydroxamic acid test over the range from 0 to 1 μmole of nucleotide was found to be proportional to the amount of TPN added and, by inference, to the amount of lactone formed. By equating hydroxamic acid color with TPNH formation the molar extinction coefficient of the iron-hydroxamic acid complex of 6-phosphogluconic acid was found to be identical to that of gluconic acid itself, as determined by using a standard solution of the δ-lactone of the latter substance. It has been reported previously (15) that these two substances do not give an equal amount of color on a molar basis in the hydroxamic acid test.

Fig. 2 demonstrates the dependence of enzymatic activity on temperature. The rate of the enzymatic reaction at 28° was linear for the first 5 minutes; it was stopped after exactly 3 minutes by the $FeCl_3$-HCl reagent of Lipmann and Tuttle (16), and the amount of hydroxamic acid formed in this interval was taken to be proportional to the specific reaction rate. Fig. 2 shows the $\log_{10}$ of the rate over the first 3 minutes determined in this manner plotted as a function of $1/T$ where T is the absolute temperature. From the slope of this plot the apparent energy of activation of the enzymatically catalyzed oxidation of glucose-6-phosphate was calculated to be

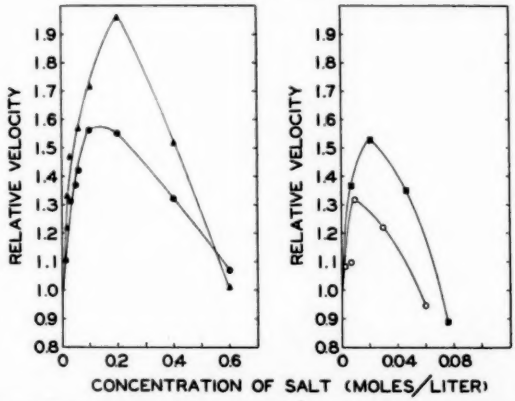

FIG. 3. Effect of various salts on enzymatic activity. The reaction mixtures contained 6.3×10^{-2} M Tris, 8.3×10^{-4} M G-6-P, 1.3×10^{-4} M TPN; pH 8.0, 25°. Enzyme (70 units per mg.) present at 0.8 γ per ml. Initial rate in the absence of added salt plotted as unity. ▲, KCl; ●, NaCl; ■, $CaCl_2$; ○, $MgCl_2$.

7070 calories per mole over the temperature range 20–37°. The Q_{10} between 25–35° is 1.48.

The possibility of a divalent metal requirement by this enzyme was suggested by Kornberg (7) who found activation by Mg^{++} in the presence of glycylglycine buffer but not in the presence of phosphate buffer. In earlier work on this enzyme (1–6) no such requirement was observed. The purest preparations of the enzyme which we have had showed approximately 70 per cent of the maximal activity in the absence of added Mg^{++}, and this activity was not reduced by having 0.025 M EDTA present in the reaction mixture. These facts make it unlikely that glucose-6-phosphate dehydrogenase has an absolute requirement for Mg^{++}. A study of the effect of various ions on the purified enzyme has now shown that it can be both activated and inhibited by a variety of univalent and divalent salts (Fig. 3). Potassium is more effective than sodium, and calcium is more effective

than magnesium. The effects observed are not on the stability of the enzyme, as they will increase the rate if added to the solution after the reaction has proceeded for some time. The effect of these ions appears to be on the affinity of the enzyme for its substrates. Thus, Fig. 4 shows that the K_m of the enzyme for G-6-P is 5.8×10^{-5} M in the presence of 0.01 M $MgCl_2$, but is 6.9×10^{-5} M in the absence of any added $MgCl_2$. Similarly, for TPN the K_m in the presence of 0.01 M $MgCl_2$ is 2.0×10^{-5} M, while in the absence of any added $MgCl_2$ it is 3.3×10^{-5} M (Fig. 5).

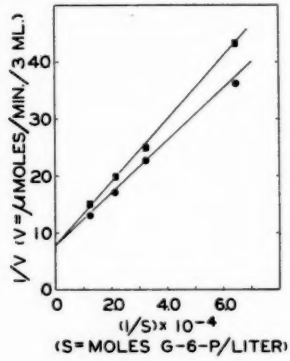

FIG. 4

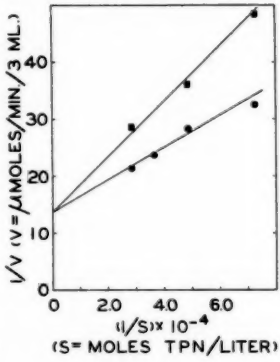

FIG. 5

FIG. 4. Effect of $MgCl_2$ on enzymatic activity at various G-6-P concentrations. The reaction mixtures contained 6.3×10^{-2} M Tris, 2.7×10^{-4} M TPN; pH 8.0, 25°. Enzyme (80 units per mg.) present at 0.5 γ per ml. ●, 1.0×10^{-2} M $MgCl_2$; ■, no added $MgCl_2$.

FIG. 5. Effect of $MgCl_2$ on enzymatic activity at various TPN concentrations. The reaction mixtures contained 6.3×10^{-2} M Tris, 8.3×10^{-4} M G-6-P; pH 8.0, 25°. Enzyme (80 units per mg.) present at 0.5 γ per ml. ●, 1.0×10^{-2} M $MgCl_2$; ■, no added $MgCl_2$.

Theorell has found (17) that the enzyme was inhibited by phosphate and that this inhibition could be reversed by TPN. We have confirmed these findings with the highly purified enzyme (see Fig. 6).

Negelein and Haas found that the enzyme is inhibited by TPNH (18). Fig. 7 demonstrates that the inhibition is competitive with TPN, and, from an analysis of the curves, the inhibition constant, K_I, for TPNH is 2.7×10^{-5} M. The enzyme is not inhibited by reduced diphosphopyridine nucleotide.

The enzyme is inhibited by D-glucosamine-6-phosphate (prepared by the action of yeast hexokinase on glucosamine (19)). Fig. 8 shows that this inhibition is competitive with G-6-P and that the K_I is 7.2×10^{-4} M. Neither mannose-6-phosphate nor *N*-acetyl-D-glucosamine-6-phosphate

(20) inhibits this enzyme. These facts emphasize the specificity of D-glucose-6-phosphate dehydrogenase.

Equilibrium of Enzymatic Reaction—Since it has been shown that the primary product of the oxidation of G-6-P is the δ-lactone of 6-phosphogluconic acid (14) and that the reaction is reversible (15), it seemed of interest to determine the approximate value of the equilibrium constant of this reaction. At pH 8.0, at which the enzyme has nearly maximal activity, the lactone decomposes quickly, but at pH 6.4 and 28° the half life

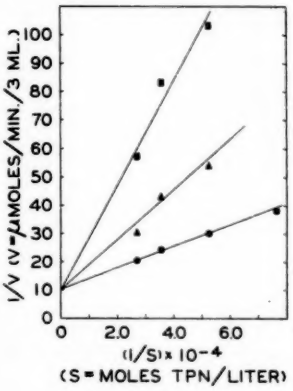

FIG. 6

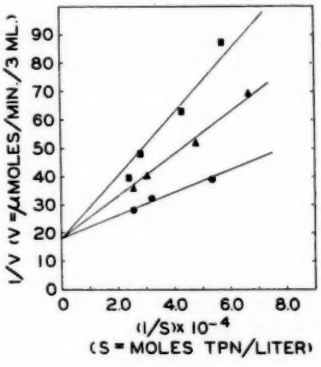

FIG. 7

FIG. 6. Inhibition by potassium phosphate of enzymatic activity at various TPN concentrations. The reaction mixtures contained 6.3×10^{-2} M Tris, 8.3×10^{-4} M G-6-P; pH 8.0, 25°. Enzyme (80 units per mg.) present at 0.5 γ per ml. ●, no added phosphate; ▲, 1.50×10^{-1} M phosphate; ■, 2.17×10^{-1} M phosphate.

FIG. 7. Inhibition by TPNH of enzymatic activity at various TPN concentrations. The reaction mixtures contained 6.3×10^{-2} M Tris, 8.3×10^{-4} M G-6-P, 1.0×10^{-2} M $MgCl_2$; pH 8.0, 25°. Enzyme (80 units per mg.) present at 0.5 γ per ml. ●, no added TPNH; ▲, 2.28×10^{-5} M TPNH; ■, 4.56×10^{-5} M TPNH.

of the lactone has been found to be 24 minutes. It seemed possible, therefore, that at pH 6.4 in the presence of a large excess of enzyme the reaction would reach equilibrium before any appreciable amount of the lactone had decomposed. Fig. 9 illustrates several such experiments. The continued slow reduction of TPN after the initial rapid reduction reflects the slow hydrolysis of the lactone. The data in Table II give the initial concentrations of the reactants in a number of similar experiments as well as the observed value of the ratio of the equilibrium constant of the reaction, K, to the hydrogen ion activity, where

$$K = \frac{(\text{TPNH})(\text{6-phosphogluconic acid } \delta\text{-lactone})[\text{H}^+]}{(\text{TPN})(\text{G-6-P})}$$

The concentration of 6-phosphogluconic acid δ-lactone at any time has been taken to be equal to the observed concentration of TPNH diminished by the amount of lactone which hydrolyzes in that interval of time. The

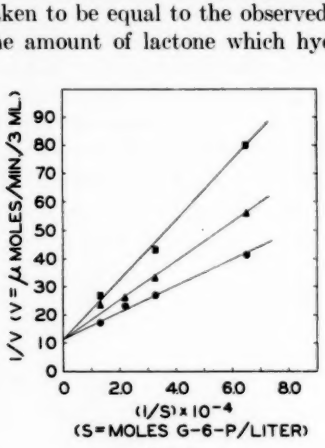

FIG. 8

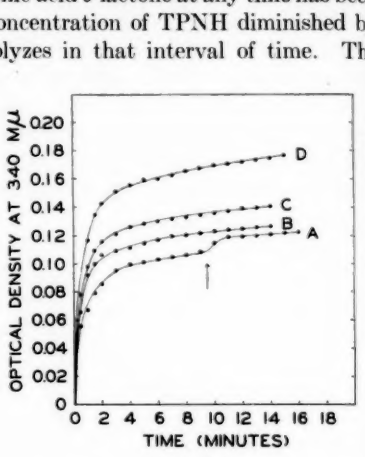

FIG. 9

FIG. 8. Inhibition by D-glucosamine-6-phosphate (Gm-6-P) of enzymatic activity at various G-6-P concentrations. The reaction mixtures contained 6.3×10^{-2} M Tris, 2.0×10^{-4} M TPN, 1.0×10^{-2} M $MgCl_2$; pH 8.0, 25°. Enzyme (80 units per mg.) present at 0.6 γ per ml. ●, no added Gm-6-P; ▲, 4.29×10^{-4} M Gm-6-P; ■, 7.15×10^{-4} M Gm-6-P.

FIG. 9. Enzymatic activity at different substrate concentrations. The reaction mixtures contained 6.3×10^{-2} M maleate buffer, pH 6.40, 1.0×10^{-2} M $MgCl_2$, TPN, and G-6-P as indicated; 28°. Enzyme (14 units per mg.) present at 43 γ per ml. Volume, 3 ml. Curve A, 3.29×10^{-5} M TPN, 2.94×10^{-5} M G-6 P. At the arrow, TPN increased to 3.95×10^{-5} M. Curve B, 3.78×10^{-5} M TPN, 2.96×10^{-5} M G-6-P; Curve C, 3.14×10^{-5} M TPN, 4.42×10^{-5} M G-6-P; Curve D, 4.42×10^{-5} M TPN, 4.42×10^{-5} M G-6-P.

TABLE II

Equilibrium Constant of Glucose-6-phosphate Dehydrogenase Reaction

Composition of reaction mixtures as in Fig. 9.

Initial TPN, μmole per 3 ml.	Initial G-6-P, μmole per 3 ml.	$\frac{K}{[H^+]}$
0.0757	0.0884	1.27
0.0986	0.0888	1.22
0.0944	0.1325	1.63
0.1183	0.0888	1.42
0.1135	0.0884	1.70
0.1135	0.1325	1.74
0.1325	0.1325	1.55

latter quantity was calculated by using the observed value of 24 minutes for the half life. The values for the equilibrium constant in Table II are the averages of those obtained at 7 and 14 minutes. Curve A of Fig. 9 represents an experiment in which, after the $K/[H^+]$ was found to be 1.22 at 8 minutes, more TPN was added, and the new $K/[H^+]$ was 1.44 at 14 minutes. The average of many determinations gives a $K = (6.0 \pm 0.7) \times 10^{-7}$ M at 28°. The corresponding standard free energy change, $\Delta F°$, is +8600 calories per mole. The data of Strecker and Korkes for the equilibrium of glucose to gluconic acid δ-lactone obtained with the glucose dehydrogenase of liver give a $\Delta F°$ of about +8000 calories per mole (21). The $\Delta F°$ for the reduction of TPN by H_2 to (TPNH + H^+) has been calculated by Burton and Wilson (22) to be +5400 calories per mole at 25°. Therefore, the $\Delta F°$ for the reaction

G-6-P = (6-phosphogluconic acid δ-lactone) + H_2

is +3200 calories per mole, corresponding to $E_0 = -0.069$ volt. At pH 6.4, $E'_0 = -0.26$ volt, while at pH 7.0, $E'_0 = -0.28$ volt. Burton and Wilson have calculated $E'_0 = -0.32$ volt for the reduction of TPN at pH 7.0.

DISCUSSION

Although a highly purified enzyme was obtained in good yield by the procedure described above, the amount of active protein present in yeast is very small. Only 9 mg. of enzyme with a turnover number of 12,000 moles of substrate per 10^5 gm. of protein per minute are present in the Lebedev juice from 200 gm. of dry yeast. Thus, the enzyme is only 0.09 per cent of the total extracted protein. The most highly purified enzyme obtained in 0.40 saturated $(NH_4)_2SO_4$ at a protein concentration of about 0.13 mg. per ml. can be precipitated only to the extent of 50 per cent by dialysis against a large volume of saturated $(NH_4)_2SO_4$. The precipitate so obtained cannot be freed of $(NH_4)_2SO_4$ without denaturing the enzyme. This fact made it impractical to obtain enough enzyme for any physical study of the protein.

The calcium phosphate gel eluate contains no 6-phosphogluconic acid dehydrogenase but is contaminated with hexose-6-phosphate isomerase and with phosphoglucomutase, as well as with hexokinase. The enzyme obtained by elution from alumina Cγ contains no phosphoglucomutase, hexose-6-phosphate isomerase, or 6-phosphogluconic acid dehydrogenase, but contains 1 part in 15 of hexokinase estimated as described above. This enzyme is purified 130 times from the Lebedev juice and may be sufficiently pure for many kinds of analytical uses.

SUMMARY

1. A preparation of D-glucose-6-phosphate dehydrogenase has been obtained from dried brewers' yeast in which the enzyme has been purified 600 to 900 times from an autolysate. The final product has a turnover number of 12,000 moles of substrate per 10^5 gm. of protein per minute at pH 8.0 and 25°.

2. The purified enzyme is free of contamination by phosphoglucomutase, hexose-6-phosphate isomerase, and 6-phosphogluconic acid dehydrogenase. Hexokinase is still present. The activity of the enzyme in the presence of glucose and ATP was 2.5 per cent of its activity in the presence of glucose-6-phosphate. A procedure is described whereby a product having one-third as much hexokinase can be obtained.

3. The enzyme has its maximal activity at pH 8.5. The apparent energy of activation of the enzymatically catalyzed oxidation of glucose-6-phosphate at pH 7.40 was found to be 7070 calories per mole between 20–37°. The Q_{10} between 25–35° is 1.48.

4. The enzyme does not appear to have an absolute requirement for any salt but can be both activated and inhibited by a variety of salts. The effect of the salt appears to be on the affinity of the enzyme for its substrates.

5. The Michaelis constants of the enzyme for TPN and for G-6-P have been determined.

6. The enzyme is inhibited by D-glucosamine-6-phosphate and the inhibition is competitive with G-6-P. The enzyme is also inhibited by phosphate ion and by TPNH. Both inhibitions are competitive with TPN.

7. The equilibrium constant of the reaction, G-6-P + TPN = TPNH + H^+ + 6-phosphogluconic acid δ-lactone, has been measured at pH 6.4 and 28°. Its value is 6.0×10^{-7} M, corresponding to $\Delta F^\circ = +8600$ calories per mole. From this value and other data the ΔF° for the reaction, G-6-P = 6-phosphogluconic acid δ-lactone + H_2, is +3200 calories per mole, corresponding to $E_0 = -0.069$ volt. At pH 7.0, $E'_0 = -0.28$ volt.

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FURTHER STUDIES ON THE PURIFICATION OF PROSTATIC ACID PHOSPHATASE*

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In a recent report a 4900-fold purification of prostatic acid phosphatase enzyme was obtained (1); however, the product was unstable and could not be crystallized. Subsequently, new techniques to simplify the purification procedure and to demonstrate the purity of the enzyme were investigated in this laboratory, and these investigations form the basis for this communication. The authenticity of crystals formed from highly purified acid phosphatase preparations is also discussed.

EXPERIMENTAL

General Methods and Materials—The source (human prostate glands), the methods of extracting the phosphatase, and the dialysis techniques for precipitation of proteins are essentially the same as those described previously (1). The assay for protein nitrogen (2), activity (3), and the unit of phosphatase activity[1] are also described by London and Hudson (1). However, in the present work the third extracts of the gland slices are pooled with the first two extracts. The supernatant fluid remaining after centrifuging the dialyzed extracts is the starting material for this study.

Fullers' Earth Adsorption—Following adsorption upon fullers' earth, quantitative elution could not be achieved, presumably because of surface denaturation. It has now been found that the phosphatase is most readily adsorbed at low pH values (3.6 to 4.5). At pH values below 4.0 there is considerable instability of the enzyme in solution, and above that value adsorption of the enzyme decreases. With the enzyme in a dilute acetate buffer (0.05 M) at pH 4.0, maximal adsorption compatible with stability is

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† Damon Runyon Senior Clinical Research Fellow.

[1] In the earlier paper the unit of activity was not clearly stated. The units of phosphatase activity per ml. are equal to micrograms of P liberated when the enzyme solution first has been diluted 250-fold, and then 1 ml. of the diluted enzyme is incubated with 4 ml. of substrate at 37° for 10 minutes.

obtained. Even at this pH, losses in activity can be detected in several hours. Tween 80, added to both the buffered dialysates before and the eluents after the adsorption, increases the recovery of enzyme. Tween 80 does not alter the adsorption or elution characteristics, but increases recoveries from both processes. Thus, the ratio of fullers' earth to dialysate is not changed in adsorbing the enzyme completely, but the recovery will be more nearly complete if Tween 80 is used. Recoveries of 100 per cent are possible if second, third, or even fourth elutions are made.

The eluting agent used with fullers' earth is 0.02 M sodium citrate at pH 7.0. This is the lowest concentration that will provide maximal elution with any fixed volume of eluting agent. Acetate buffer at pH 5.8 (0.02 M) is a weak eluent. Sodium bicarbonate elutes the enzyme, but, on standing, the pH of the eluate rises and denatures some of the enzyme. With the use of bicarbonate, soluble material is leached from the fullers' earth. A precipitate forms, and enzyme activity is lost (presumably from surface denaturation) whenever the pH of the bicarbonate eluates is lowered to a value which usually has been found to stabilize the enzyme.

After the enzyme is adsorbed on fullers' earth, a distilled water wash is omitted. Distilled water elutes some activity. A wash can be made with dilute acetate buffer at pH 4.0.

The amount of adsorbent required depends on the quantity of protein in solution, and the amount of fullers' earth will vary as much as 3-fold according to its source. To obtain the maximal recovery of enzyme, only about 25 per cent of the activity is adsorbed. In Fig. 1, a typical adsorption curve, there is a region of intense adsorption of the enzyme between about 25 and 80 per cent adsorption. In this range the enzyme is selectively adsorbed upon fullers' earth, the rest of the curve (80 to 100 per cent adsorption) resembling a normal adsorption isotherm for a single substance. This suggests that the enzyme adsorption begins at the 25 per cent level with little competition from other proteins for the active sites of the fullers' earth. Minimal competition occurs at about 60 per cent adsorption and continues till the adsorption is nearly completed. Therefore, when the adsorption is about 25 per cent, a purified fraction in the liquid phase is obtained. In addition, a minimum of enzyme is exposed to surface denaturation. It is desirable to adsorb the enzyme only partially. The best purification does not coincide with the greatest recovery. Better purifications are obtained when about 50 per cent of the initial activity is adsorbed. The adsorbed material can be selectively eluted and added to the non-adsorbed enzyme fraction.

A typical fullers' earth adsorption-elution is as follows: To 1800 ml. of dialyzed extract, containing 32 phosphatase units per ml. and 3.6 mg. of protein per ml., were added 200 ml. of 1.0 M acetate buffer, pH 4.0, contain-

ing 5 per cent Tween 80. Then 70 gm. of fullers' earth were suspended in the buffered solution with thorough mixing for 5 minutes. The quantity of fullers' earth used had been estimated to provide 50 per cent adsorption of enzyme by testing with smaller quantities. The suspension was sedimented at 3000 × *g* at 20° for 15 minutes. The supernatant fluid was removed and saved, and the sediment, which consisted primarily of protein adsorbed on fullers' earth, was resuspended in 1.0 liter of 0.02 M sodium citrate, pH 7.0, containing 1 per cent Tween. The mixture was stirred for 5 minutes, and most of the adsorbed enzyme (about 50 per cent of the dialyzed extract) was eluted. The suspension of fullers' earth protein then contained very little enzyme, as was shown by centrifuging as before and

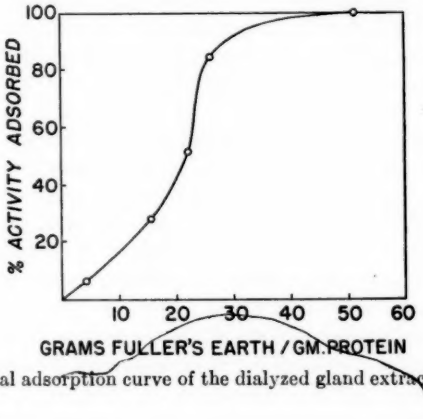

FIG. 1. A typical adsorption curve of the dialyzed gland extracts at pH 4.0 in 0.05 M acetate buffer.

assaying the supernatant fluid. The supernatant fluid from the adsorption was combined with the eluted enzyme, and the combined fraction contained 15 phosphatase units per ml. and 1.0 mg. of protein per ml. in 2900 ml. of solution. The recovery in this pooled fraction was almost 80 per cent. A crude estimate suggested that about 5 per cent of the original enzyme was in the solution wetting the fullers' earth, but it could not be recovered. A second elution with more of the same eluting agent caused the desorption of more enzyme, but it was less pure than the starting material, and was discarded with the fullers' earth. As much as a 4-fold purification was in some instances obtained by this procedure.

Preparation for Salt Fractionation—Prior to salt fractionation of the acid phosphatase preparation after fullers' earth adsorption, it is necessary to concentrate the protein and enzyme and to remove the Tween 80. The consequence of not removing the Tween is that the enzyme will not fractionate properly. Tween separates as an oil at high salt concentrations.

Under these conditions protein and activity will adsorb on the oil even at concentrations of salt far below that which is normally required to salt out the enzyme.

The enzyme solution after elution is immediately adjusted to pH 5.3 with appropriate acetate buffer. The protein is concentrated by a stream of filtered hot air blown over a large surface area of the combined fullers' earth fractions, the solution temperature remaining below 20°. Tween concentration is not allowed to exceed 5 per cent during the evaporation process lest the enzyme begin to denature. During the evaporation the pH of the solution is kept below 5.8 by the addition of 0.2 M acetic acid if necessary. After the evaporation, the solution, which has been concentrated 4-fold, is centrifuged at 3000 × *g* to remove more fullers' earth and other inert material which has precipitated.

Lyophilization cannot be used to remove water because freezing the Tween 80-aqueous mixture inactivates the enzyme.

Ammonium Sulfate Fractionation—For purification the Tween is separated from a 40 per cent saturated $(NH_4)_2SO_4$ solution. This is done by adding 2 volumes of saturated $(NH_4)_2SO_4$ to 3 volumes of Tween-enzyme concentrate. By adding more saturated $(NH_4)_2SO_4$ solution, the new solution is then made 55 per cent saturated; this step removes only inert protein. The enzyme is then almost quantitatively precipitated by bringing the solution to 68 per cent saturation.[2] Between each step an overnight waiting period at room temperature is necessary. It is recommended that a separatory funnel be used in these processes, and that the packing of protein sediments be done by high speed centrifuging. The sedimented protein containing the enzyme is taken up in distilled water. The recoveries of activity in this step are usually as much as 80 per cent, and the purification is often over 5-fold. This compares most favorably with the original procedure (1).

Ion Exchange Chromatography; Preparation of Resin—Amberlite XE-69 (Rohm and Haas)[3] is suspended in 0.1 M HCl in a pear-shaped separatory funnel, the liquid height being 45 cm. Particles settling in 30 seconds are

[2] The reasons for adding $(NH_4)_2SO_4$ as described are provided by the following: When a volume of saturated $(NH_4)_2SO_4$ solution at room temperature is added to 2 volumes of the Tween-enzyme concentrate, the Tween separates from solution and floats to the top. This separation is quantitative, as further addition of $(NH_4)_2SO_4$ will show. 2 volumes of the saturated salt solution, added to 3 volumes of Tween-enzyme concentrate, will remove both the Tween and some inert protein. 11 parts of saturated $(NH_4)_2SO_4$ added to 9 parts of Tween-enzyme will precipitate almost all protein, including the enzyme with the Tween. Two precipitates result, one rising and the other settling.

[3] The resin as obtained contains more than 50 per cent of material smaller than 300 mesh. The resin is a strong cation exchanger with a cross-linking of 7 to 10 per cent. This is similar to Dowex 50.

removed and discarded, and this procedure is repeated three times. The resin settling in 2.0 hours is collected and resuspended in 0.1 M HCl. Again, the resin settling in 2.0 hours is collected (cloudy supernatant fluid is discarded). The sediment is resuspended in 0.1 M NaOH, and the portion that settles in 2.0 hours is collected. The NaOH suspension is repeated, and all particles settling overnight are collected. The resin is washed repeatedly with 0.2 M acetate buffer (either pH 4.5 or 5.0) until the buffer pH is maintained. Care is taken in washing to recover all the fine particles.

The column is constructed by adding a slurry of the resin, in acetate buffer, up to the height desired, and is washed overnight by slow percolation with the acetate buffer.

After a chromatogram is completed, the resin is cleared of any protein remaining on the column by refluxing the resin for 2 hours in M HCl. The resin is further regenerated by bringing it in contact with buffer to return it to desirable pH.

Typical Chromatogram—Ammonium sulfate-fractionated material is centrifuged until it is clear, and is then dialyzed against acetate buffer (0.2 M; pH 5.0). About 60 ml. of dialyzed enzyme containing 375 phosphatase units and 5 mg. of protein per ml. are placed on a 4.4 × 15 cm. column. Toluene is layered over the enzyme solution to increase the flow rate during the adsorption, but is not allowed to come in contact with the resin. About 50 ml. of the same acetate buffer are allowed to follow the enzyme through the column as a wash. The eluent, 0.2 M acetate buffer, pH 5.85, is then carefully added when the head of the wash is reduced to a fraction of a cm. The flow rate is fixed by adjusting the height of the eluent reservoir. The flow rate during the elution as well as the wash and adsorption is approximately 24 ml. per hour. As the effluent percolates through the column, fractions are allowed to collect at 30 minute intervals. The results of the chromatogram are shown in Fig. 2.

Column Capacity—As previously indicated (4–6), the quantity of ion exchange resin required to adsorb proteins is disproportionately large. It is out of proportion in terms of other adsorbents acting on proteins (7), and in terms of resin required to chromatograph small molecules. Sober *et al.* (4) indicated that the particle size may be the determining factor in the adsorptive capacity. To test this hypothesis, resin already sized as described was sized further. Particles settling in acetate buffer at pH 5.0 were collected in three fractions based on the length of time of settling, as shown in Table I. From these fractions three columns 1 × 4 cm. were prepared. Identical enzyme solutions, each containing 6 mg. of protein and 500 units of activity, were added to each of the columns. They were washed with equal volumes of acetate buffer, pH 5.0, to remove unadsorbed

protein. The columns were eluted with acetate buffer, pH 5.85. Table I shows the variation in adsorptive capacity with size by indicating percentile recoveries of the original solution.

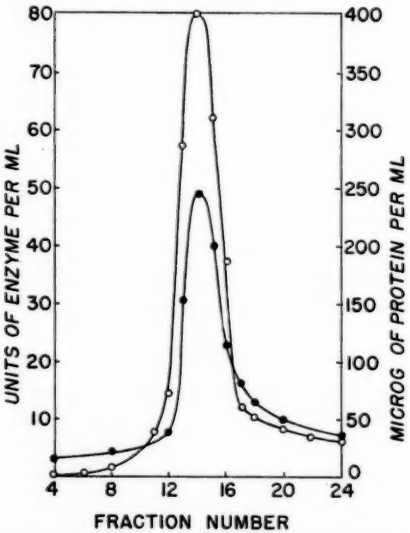

FIG. 2. A typical chromatogram of the type used in large scale purification of the enzyme. ○, activity curve; ●, protein curve. For the details, see Table II, Chromatogram VIII.

TABLE I

Variation of Adsorptive Capacity with Particle Size

Settling time	Dry weight, resin	Activity washed through, pH 5.0	Activity washed through, pH 5.85	Total recovery
min.	*gm.*	*per cent*	*per cent*	*per cent*
0– 5	2.1	63	22	85
5– 30	2.2	35	38	73
30–120	2.0	10	70	80

Considerable variation in adsorptive capacity was found in different batches of XE-69. This variation could be correlated with particle size. Used resin was allowed to settle for 1.5 hours, the supernatant fluid containing some fine particles being discarded. The resin was observed to lose adsorptive capacity; each resin must be tested for capacity prior to use in purification. Capacity in elution chromatography is not determined alone by how much will be adsorbed, but is influenced by properties which

promote fractionation as well. As a rule, less than one-fourth of the total adsorptive capacity should be used in obtaining a chromatogram.

Adsorption-Elution—It is noteworthy that the isoelectric point of the enzyme is at pH 4.5 (8). The enzyme should have a greater affinity for the resin at pH 4.5 than at 5.0, but differences in the chromatogram were not apparent when the buffers were interchanged for adsorption. With 0.2 M acetate buffer, it was found that at pH 5.3 the enzyme preparation would not produce a chromatogram, and it could not be used as an eluent when enzyme was adsorbed at a lower pH. At pH 5.4, elution began, but recoveries were poor (about 20 per cent). The enzyme recovery gradually increased up to about 70 per cent and leveled off at pH 5.7. The enzyme was eluted over a narrower band as the pH of the eluent was increased, and resulted in less trailing in the chromatogram. Raising the pH increases the recoveries but makes separation of zones more difficult. Maximal recovery and good separation of zones for purification are obtained at pH 5.85 (Fig. 2).

Various other features of the chromatography are summarized in Table II. It was observed that a preliminary wash with a buffer not sufficiently high in pH to elute produces a good maximal purification, but indications are that prolonged exposure to the column increases losses on purification. One reason for the failure to recover more than half of the protein, and more than 85 per cent of the activity in the best chromatograms, is indicated in Fig. 3, which shows a leveling off at the end of the chromatogram. A low but almost constant level of protein and activity in the effluent seems likely. Tween 80 apparently provides no surface protection for the enzyme on the column, as indicated by low recoveries. Citrate does not increase elution from the ion exchange column, contrary to the findings with fullers' earth. Acetate was used in all elutions except in the first case shown in Table II.

General Purification—A purification of the enzyme was attempted with the new techniques described above. Table III summarizes the essential purification data.

Enzyme preparations lose their foaming power in the passage through the fullers' earth purification procedure, and are not then suitable for foam fractionation if subsequently treated with ammonium sulfate. The concentration of protein required to produce a sufficiently stable foam for fractionation is too high for the effective partitioning of proteins in froth. Enzyme preparations purified by the older technique (foaming) cannot readily be purified by chromatography beyond the specific activity indicated in Table III for the chromatographic step. Repeated chromatography did not produce purification better than the limiting values shown in Tables II and III.

Tests of Purity—If a pure enzyme is denatured, identical rates for the

TABLE II

Summary of Chromatography Adsorption-Elution Data

Chromatogram No.	Eluent pH	Recovery	Column size	Volume		Highest purity†	Remarks
				Fraction A	Fraction B*		
		per cent	*cm.*	*ml.*	*ml.*		
I	6.5	64	1 × 15	1.8	5	9	Citrate eluted, Tween 80
II	6.0	33	2 × 17.5	2.0	64	10	Adsorbed, pH 4.5, Tween 80
III	6.0	79	2 × 15	3.2	38	9	No wash
IV	6.0	67	1 × 15	1.0	14	9	Rechromatogram of III
V	5.7	70	1 × 15	1.4	8.4	7	Adsorbed, washed; pH 4.5
VI	5.5	25	2 × 14.5	3.0	33	9.4	Prolonged wash; pH 5.3
VII‡	5.6	48	2 × 8	2.6	23	12.3	" "
VIII§	5.85	85	4.4 × 15	12	96	10	Washed; pH 5.0
IX	5.4	20	1 × 8	2			
X	6.0	65	1 × 8	2			

* Volume through before enzyme appears, after adding eluent.

† An arbitrary specific activity, based on 2.3 as the starting material (fullers' earth, ammonium sulfate fractionated).

‡ Same as Fig. 3.

§ Same as Fig. 2; used as the method of purification.

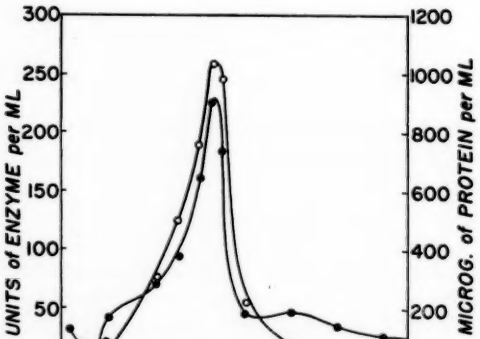

FIG. 3. A chromatogram in which buffer at pH 5.3 has been used to spread out protein prior to elution. ○, activity curve; ●, protein curve. For the details, see Table II, Chromatogram VII.

loss in soluble protein and activity will be obtained; *i.e.*, all enzyme molecules and all protein molecules are identical. Loss of solubility is frequently the criterion observed in protein denaturation processes. If an enzyme preparation is impure, the closeness of fit of denaturation curves as measured by activity and solubility can serve as a measure of purity. Because of the possibility that a combination of impurities of varying stability may approximate the activity denaturation curve, assurance that this is not likely is obtained by varying conditions of denaturation.

Since the enzyme was purified by foaming (1), it is presumed from the earlier data that as a protein it is relatively stable toward surface denaturation. If phosphatase is shaken in a protein solution, the protein could be

TABLE III

Summary of Purification of Phosphatase

Process	Activity	Concentration	Purification fold	Yield
	units per ml.	*mg. protein per ml.*		*per cent*
Glands*			1.0	(100)
Combined extracts*	42	8.6	21.5	76
Pooled dialysates*	32	3.6	40.6	58
Fullers' earth	15.5	1.0	70	46
Air evaporation	62	4.0		42
Ammonium sulfate	390	5.0	350	29
Dialysis				
Chromatography	115	0.29	1780†	20

* Data and method taken from an earlier paper (1).

† This is the purification obtained after the combined eluates had been in a bottle for several weeks and some protein had denatured.

expected to denature before the phosphatase unless the preparation is pure or the less stable proteins have been removed by fractionation. Enzyme purified through chromatography was shaken mechanically with a few drops of toluene to cause denaturation. Measurement of activity and the soluble protein indicated no greater than 30 per cent loss in specific activity during the course of denaturation. A similar result was obtained when the shaking was done in air to form a foam to produce denaturation. These observations suggest a high degree of purity.

In this laboratory (9) it was demonstrated that NaF, a highly specific inhibitor for the prostatic acid phosphatase, also stabilizes the enzyme against heat denaturation. The concentration of NaF that causes maximal inhibition coincides with the concentration of that salt required for optimal thermal stabilization. The facts suggest a complex formation of fluoride with the enzyme and that this complex is inactive and relatively

heat-stable. Since the reaction with fluoride is specific, the enzyme may be considered thermally stabilized relative to the other proteins present. The same chromatographed preparation was subjected to heat denaturation by incubating the enzyme at 56.6° for 2 hours in pH 5.5, 0.2 M acetate buffer, containing 0.002 M NaF, and at 56.6° in the same buffer without fluoride. Analysis of the soluble protein showed a 33 per cent loss in specific activity and a 50 per cent loss in total activity in the fluoride-treated

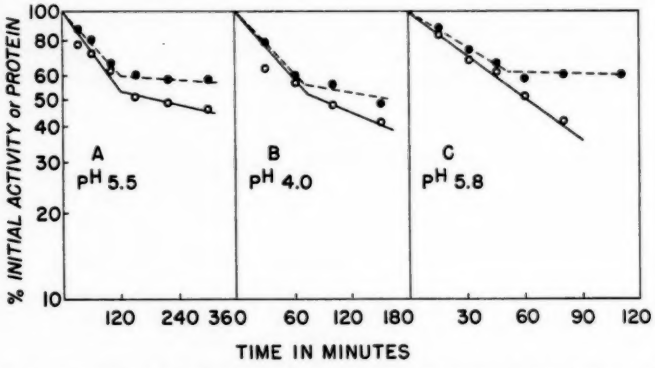

FIG. 4. ○, residual activity in solution. ●, residual protein in solution. Curves of *A* and *C* were obtained by removing the hot mixtures from their 50.3° bath and centrifuging cold. Aliquots of clear supernatant fluid were measured for protein nitrogen and activity. The denatured protein of *B* was soluble at pH 4.0. Aliquots of *B* removed from the 50.3° bath were mixed with equal volumes of buffer, pH 5.8, and then centrifuged cold before assaying. The denatured protein precipitated, and the soluble protein was determined. Acetate buffers used were at 0.18 M. The apparent breaks of activity curves in *A* and *B* are real, and caused by a slight lowering of bath temperature from the 50.3° used during the heating period. The breaks in the protein curves are not due to temperature variations alone, but probably represent, as in the curve in *C*, an alteration in composition when less stable protein has been spent. Initial protein concentrations were 2.5 to 2.8 mg. per ml.

sample. Without the fluoride, the destruction of activity was about 50 per cent in 10 minutes (10).

By utilizing available data (10) concerning the first order heat denaturation constants of this enzyme, three samples of the preparation were heat-denatured, at 50.3° under controlled conditions. Three sets of denaturation curves for protein and activity are presented in Fig. 4. Approximate rate constants derived from them fit close to the values previously obtained with less pure enzyme. Corresponding activity and protein curves indicate the degree of purity, and it is estimated from them that the enzyme protein would constitute 30 to 60 per cent of the total.

The fact that purification steps, including combinations of foaming and chromatographic methods, fail to increase specific activity indicates an already high degree of purification. Chromatography (see Fig. 2) produces a highly symmetrical single peak, and denaturation procedures do not alter the specific activity grossly; this indicates the presence of a large fraction of a single component.

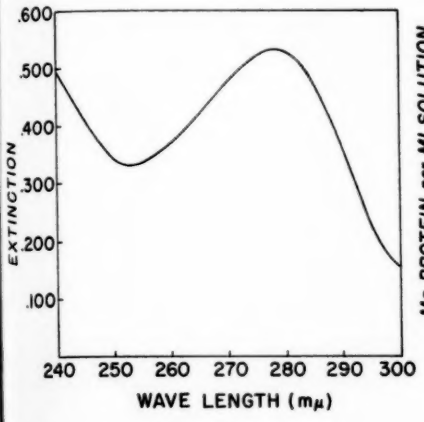

FIG. 5

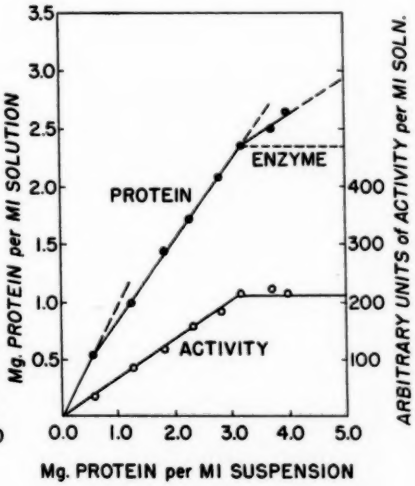

FIG. 6

FIG. 5. Ultraviolet absorption curve of protein obtained from a preparation purified through chromatography. Concentration was 0.190 mg. of protein per ml. The preparation was dialyzed against distilled water before the curve was obtained (final pH, about 6).

FIG. 6. Solubility curve of prostatic acid phosphatase preparation; temperature, 25.0°. Ammonium sulfate concentration of 54.5 per cent saturation in 0.1 M acetate buffer at pH 5.0 was maintained by adding concentrated enzyme preparation in buffer and compensating for salt concentration by adding the appropriate amount of saturated $(NH_4)_2SO_4$.

A solubility curve appeared worth while for analyzing the component system. The technique used was essentially that described by Northrop *et al.* ((11) p. 288). To conserve purified enzyme in making protein analyses, the difference between absorption values of the preparation at 278 and 300 mμ in ultraviolet light was used. Small samples used for the ultraviolet absorption were diluted before the measurements were made. From the diluted samples aliquots were withdrawn and assayed for activity. The absorption curve in ultraviolet light for protein of the chromatographed preparation is shown in Fig. 5. The solubility curve obtained is shown in

Fig. 6. It should be noted that there are two breaks in the solubility curve, indicating at least three components. An analysis of the slopes and the coincidence of the disappearance of increasing activity in solution with one of the breaks allow the estimation of 36 per cent enzyme protein in the preparation. This quantitative estimation is dependent on the constant ratio of ultraviolet absorption to protein weight.

Crystallization—Dialyzed enzyme solution was concentrated by lyophilization to 5 per cent protein,[4] adjusted to pH 5.0 with acetate buffer, and

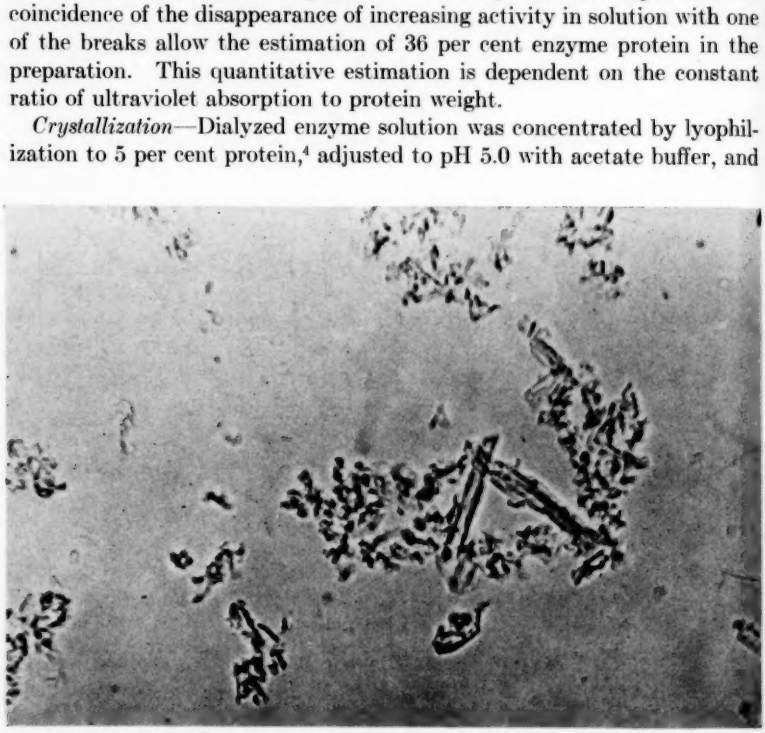

FIG. 7. Crystals obtained from a purified prostatic acid phosphatase preparation (× 1350). Photographed cold (about 5°).

solid $(NH_4)_2SO_4$ was added. The solution was kept at −5°, and when, on addition of $(NH_4)_2SO_4$, a precipitate resulted which, on stirring, persisted, a small drop of water was added, and the solution cleared. Overnight, the solution was allowed to reach 4° gradually. In the morning a precipitate

[4] Prior to the final concentration by lyophilization, it was found convenient to obtain an initial concentration of purified preparations with $Ca_3(PO_4)_2$ gel and citrate as eluent, as described earlier (1). The preparation of $Ca_3(PO_4)_2$ gel used previously (1) was not correctly described. The NH_3 was actually added prior to the addition of Na_2HPO_4, rather than afterward. By adding Na_2HPO_4 (to $CaCl_2$) prior to NH_3, a gel having 10 times the adsorptive capacity, but less specificity for purification, resulted. The gels used to concentrate dilute enzyme solutions were prepared by adding Na_2HPO_4 before NH_3.

appeared which suggested crystals. On stirring there was a sheen associated with the precipitate, and, in general, the moving precipitate had a silky appearance. The precipitate was sedimented in the cold and its specific activity was found to be much lower than the original activity. The $(NH_4)_2SO_4$ content of the supernatant fluid was 55 per cent of saturation. The entire crystallization process was repeated on the supernatant fluid by raising the $(NH_4)_2SO_4$ concentration to 60 per cent of saturation with more solid salt. In the next precipitate the crystals had the same gross appearance. When sedimented and separated from the supernatant fluid, the crystals had a specific activity about equal to the starting material. At low magnification the crystals could not be detected. At 400 ×, only about 5 to 10 per cent of the precipitate could be recognized as crystalline. At the higher magnification shown in Fig. 7, more than 10 per cent of the solids seen was crystalline. An enzyme preparation, purified by foaming and having a specific activity approximately equal to the chromatographed preparation used in this work, produced similar crystals.

DISCUSSION

It remains to be proved that the crystals seen contain the enzymatic activity. That the preparation lost considerable activity during the attempts at crystallization may account for the failure of the crystals and amorphous precipitate to exhibit an increased specific activity. The general picture of poor crystallization properties coupled with denaturation are reminiscent of the experiences reported with crystalline diphtheria antitoxin ((11) p. 192). The general consideration that purified material denatures rapidly may account for the complete failure of crystallization with a purer preparation (1). It was much less stable. The fact that crystals can be formed is an indication that there are few components, or at least a few components making up the bulk of the preparation.

The 36 per cent purity value obtained is not necessarily quantitatively correct, since the measurements are based on the presumption that the three components observed in the solubility curve have the same optical densities in ultraviolet absorption. The earlier purification (1) of 4900-fold is about half again as great as the theoretical maximum of 3100-fold, calculated from solubility and from the specific activity of 1780-fold purified material, which had already deteriorated 40 per cent in specific activity on standing. All the evidence available does indicate that the earlier preparation was almost completely pure.

Each of the two purification procedures has advantages. The newer method is simpler in working time requirements and produces a somewhat less pure preparation, but one which is relatively stable. It loses only 10 to 15 per cent of its activity each month. The other preparation decom-

poses about 4 times as fast. However, both preparations deteriorate at an accelerated rate on standing in ammonium sulfate solutions.

In combining purification methods, the finding that preparations treated with fullers' earth do not foam readily helps to clarify certain data. It seems that the enzyme is not very surface-active, but, under proper conditions, high purity or dilution, it is susceptible to surface denaturation. The use of Tween apparently serves to protect the enzyme by keeping it away from a destructive fullers' earth interface. The more active proteins (those which readily produce foams) are adsorbed by fullers' earth even in the presence of Tween. The enzyme adsorption onto the fullers' earth seems to be regulated by charging conditions; the fullers' earth is a negatively charged colloid, and the enzyme is positively charged at pH 4.0. The Tween in this case probably forms a fine barrier between the surfaces of the two particles, but not great enough to interfere with the proximity of the charges during adsorption.

Recently, Boman (12) described the gradient elution of the phosphatase enzyme of seminal origin from Dowex 50. A 5-fold purification was attained. Semen, as a starting material, is poorer than the enzyme preparation used in our chromatography. The specific activity of phosphatase in semen is about equal to that of the crude extracts of the glands. Although the chromatographic method is more tedious, it produces a better purification. Generally, there is a close similarity of characteristics of the enzyme as determined by the two different methods. Boman found that the activity starts to elute at pH 5.4 and that most of it is eluted at pH 5.5 and higher. The general characteristic that the enzyme travels ahead of most of the protein on the column is also observed. Our recoveries compare favorably with his 75 per cent recovery. Room temperature (25°) used in our work gave as good recoveries as the 4° environment used by Boman. He indicated that he estimated protein by ultraviolet absorption, although nitrogen was measured directly, for peaks. In this laboratory it was found that crude extracts of prostate gland contained no peak in the 280 mμ region. It began to appear only after considerable purification. Therefore, any method of measurement for aromatic amino acid residues in the early steps of purification may lead to gross errors in calculations of specific activity. Boman mentioned that use of the Folin reagent gave unreasonable figures.

SUMMARY

A new method of purifying the prostatic acid phosphatase has been presented. Previously, fullers' earth was found to be unsuitable, but now this surface-denaturing agent can be used for purifying the enzyme. The

process is made feasible by protecting the enzyme with a non-ionic surface-active agent.

The ion exchange properties of the enzyme are presented. A highly purified product was obtained by chromatography.

Solubility, heat denaturation, chromatography, purification characteristics, and the formation of crystals were used to demonstrate the purity of the preparations produced in this laboratory. The crystals observed and reproduced here may be the enzyme.

We wish to thank Dr. John M. Reiner for his timely suggestions and encouragement. We also owe thanks to Miss Barbara Wigler for her technical assistance.

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PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF AN UNUSUAL BENCE-JONES PROTEIN*

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Bence-Jones proteins are a class of molecules apparently found in all cases of multiple myeloma (1), which are characterized by unique solubility properties (2). They have been reported to possess molecular weights from 24,000 to 90,000, to possess widely divergent diffusional, electrophoretic, and ultracentrifugal properties, and at times to appear in the urine of a given patient as a mixture of several proteins (3–6). It was of interest to study carefully a unique Bence-Jones protein which under certain conditions exists as a relatively homogeneous physical entity and under others gives evidence of several distinct components. The protein showed some departure from the usual solubility properties of this type of molecule, which lends additional interest to its careful characterization.

EXPERIMENTAL

The protein in question was separated from the urine of a patient with symptoms of multiple myeloma.[1] The serum of this individual was reported by the clinical laboratory to have an albumin-globulin ratio of 4.3 and the urine to possess an albumin content of approximately 1.5 per cent. However, in more dilute solution and at slightly acid pH values, the urine gave the heat-solubility reactions of the usual Bence-Jones protein. The electrophoretic pattern of the serum proteins in diethyl barbiturate buffer, pH 8.6, 0.1 ionic strength, as shown in Fig. 1, has an albumin-globulin ratio of 0.76 and indicates a γ_1-hyperglobulinemia. Examination of the nondialyzable urinary components under similar conditions revealed a relatively homogeneous protein, possessing the electrophoretic mobility of a γ_1-globulin.

The proteins in pooled 24 hour urine collections were precipitated by the addition of solid $(NH_4)_2SO_4$ to 60 per cent saturation at 25°. The precipitate was removed by filtration, and the resultant paste was dialyzed. The small amount of insoluble material formed was discarded, and the

* This work was supported in part by a grant from the United States Public Health Service.

[1] We are indebted to the Medical Service for making available blood and urine samples of this patient.

protein was then recovered by lyophilization. Approximately 28 gm. per 24 hour urine collection were obtained. The electrophoretic patterns for this material in 0.1 ionic strength buffers at pH 8.6 and 3.9 are also given in Fig. 1.

The protein thus obtained was further fractionated to remove the material which appears at pH 8.6 as a small and faster migrating electrophoretic component of the main peak. This contaminant is more readily seen in the experiment at pH 3.9 of Fig. 1 as the material between the salt boundary and the major component. A 1 per cent solution of it in 0.02 ionic strength NaCl at pH 6.3 was cooled to 0°, and precooled 50 per cent ethanol was added to a concentration of 15 per cent, final temperature −6°. The precipitate formed was discarded and the supernatant solution brought to 25 per cent ethanol. The resultant precipitate was removed and suspended in an ice-water mixture. $NaHCO_3$ and NaCl were added

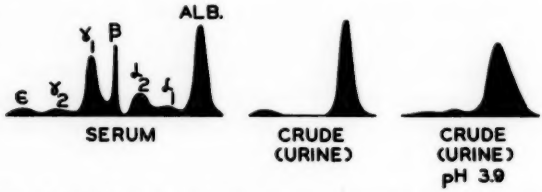

FIG. 1. The electrophoretic patterns of serum and Bence-Jones proteins at pH 8.6 (except where indicated). The duration of experiments was 180 minutes.

to bring the pH to 7.4 and the ionic strength to 0.02. An equal volume of precooled ethanol containing 0.02 M NaCl was added. The precipitate was recovered by centrifugation and was then twice precipitated at pH 7.4 under the conditions just described. The protein was then dried by lyophilization. This treatment sufficed to remove the contaminant components of the protein fraction isolated from urine and small traces of serum albumin which could be detected in the crude urinary protein fraction by immunological tests.

Solutions of this material were exhaustively dialyzed against distilled water. Weighed aliquots of the solution were then dried to constant weight at 105° to determine the protein concentration. Such standardized solutions were used for various organic and physicochemical determinations.

Results

Electrophoretic Experiments—The purified protein had a single symmetrical boundary in 0.1 ionic strength buffers from pH 8.6 to 4.5. While this protein appeared to be similar in gross solubility properties to the

Bence-Jones protein G, described briefly by Putnam and Stelos (6), it did not show two major components upon electrophoresis in buffer at pH 8.6. Below pH 4.5 a second faster migrating shoulder appears. It can be seen from Fig. 1 that the major component of the crude urinary protein fraction is asymmetric in buffer at pH 3.9. This asymmetry is usually more pronounced, and often a well defined component may be partially resolved from extensively fractionated material. Electrophoretic experiments in alkaline buffers after preliminary incubation at pH 4 show the presence of only a single component, which indicates that the second component formed at acid pH is not an irreversible denaturation product. As shown by the data of Fig. 2, the protein has an isoelectric point near 5.25 at 0.1 ionic strength. The slope of the pH-mobility curve in this region is -1.18

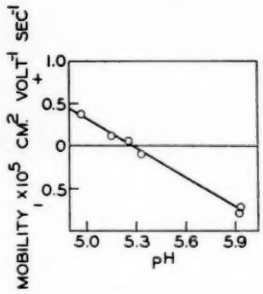

FIG. 2

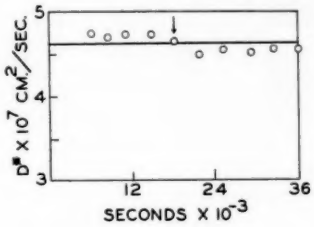

FIG. 3

FIG. 2. The electrophoretic mobility as a function of pH.

FIG. 3. The apparent diffusion constant as a function of time. The current was reversed at the point indicated by the arrow.

$\times 10^{-5}$ sq. cm. per volt per second per pH unit. The protein has a high degree of electrical homogeneity when studied by the reversible boundary spreading method of Alberty (7) at pH 5.25. In these experiments the initial boundary was sharpened by the method of Kahn and Polson (8). The data of Fig. 3 show that there is no apparent drift of the diffusion constant with time of electrophoresis at the isoelectric point. Thus this protein is made up of molecules which are essentially homogeneous from an electrical standpoint.

Velocity Sedimentation Studies—In these experiments the Spinco ultracentrifuge at gravitational fields near 2.5×10^5 g was employed. A single component is seen in buffers near pH 4, but above pH 5 two components are evident. The average sedimentation velocity for twelve experiments with the homogeneous system gave an s_{20w} of 3.50×10^{-13} cm. per second. The second component seen above pH 5 has an s_{20w} equal to 5.23 S. When a solution containing two components is returned

to pH 4, the faster sedimenting component disappears. It would appear that the latter is a dimer which forms above pH 5. Sedimentation diagrams of the protein at neutral and acid pH conditions are presented in Fig. 4. No apparent dependence of the sedimentation constant on protein concentration is indicated by the data of Fig. 5, which suggests that the molecule is quite symmetrical.

FIG. 4. Sedimentation patterns in acetate-NaCl buffer, 0.2 ionic strength, pH 3.9 (upper diagram), and in potassium phosphate buffer, 0.2 ionic strength, pH 7.4 (lower diagram). The interval between successive exposures is 32 minutes.

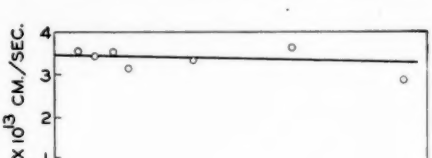

FIG. 5. The effect of protein concentration on the sedimentation velocity

Diffusion Experiments—The diffusion constant of this protein was measured at pH 3.9, at which point ultracentrifuge studies indicated that essential molecular homogeneity obtains. The value, as measured by the height-area method, was 9.65×10^{-7} sq. cm. per second. Experiments run near the isoelectric point and at neutral pH gave some deviations in values, but they were always considerably lower than the results obtained near pH 4. The lower and variable results are probably due to the presence of variable amounts of the heavier molecular weight material seen in the ultracentrifuge experiments at the higher pH values.

Density Determinations—The apparent specific volume ($V_{sp.}$) was deter-

mined on solutions of 8.424, 4.269, and 2.272 per cent protein. The corresponding values of $V_{sp.}$ were 0.720, 0.718, and 0.716 respectively. The value extrapolated to zero protein concentration is near 0.715. Values of 0.749 have been previously reported for two Bence-Jones proteins (9). The rather high density for this protein is surprising in view of its very low carbohydrate content.

Molecular Weight—By using the sedimentation and diffusion constants obtained near pH 4 and the $V_{sp.}$ of 0.715, a molecular weight near 31,000 may be readily calculated. This is somewhat lower than the values previously reported by Svedberg and Pedersen (9) and considerably more so than the values near 43,000 reported by Putnam and Stelos (6). However, Rundles, Cooper, and Willett (5) have indicated that the molecular weights of Bence-Jones protein may range from as low as 24,000 up to 90,000. Both groups of workers (6, 5) assumed $V_{sp.}$ values near 0.75 in their calculations. In view of our much lower value, it is possible that their molecular weights may be from 10 to 15 per cent lower in some cases.

From the molecular weight and $V_{sp.}$ data, a frictional ratio close to 1 may be calculated for the protein in buffer of pH 4, 0.2 ionic strength. Thus the molecule must be essentially spherical. This is in agreement with the previous failure to observe any variation of the sedimentation constant with concentration.

Other Studies—The protein contained 15.94 per cent nitrogen determined by a micro-Kjeldahl method. No hexosamine could be detected by the method of Hewitt (10) after hydrolysis for 6 hours at 100° in 2 N HCl. Only 0.24 per cent sugar was found by the orcinol method of Sørensen and Haugaard (11).

The protein contained nearly 0.25 per cent methionine.[2] This is equivalent to 1 residue of this amino acid per 60,000 gm. of protein, or sufficient for only half of the molecules to possess this amino acid. Recent amino acid analyses of Bence-Jones proteins indicate that they are usually very low in or lack methionine (12–15). Ågren (15), however, has studied one preparation that would contain 1 mole of methionine per 37,000 gm. of protein.

DISCUSSION

The Bence-Jones protein studied in this work has been found to be an electrically homogeneous substance above pH 5. However, at this condition it contains two molecular components. Thus under conditions in which the molecule is electrophoretically homogeneous, it is molecularly heterogeneous and vice versa.

While its physical properties fall within the range reported for other

[2] We wish to thank B. E. Kline for carrying out this analysis.

Bence-Jones proteins, its property of coagulating at about 60° near neutral pH differentiates it from the usual molecule. Putnam and Stelos (6) also found a similar protein in the urine of a patient with multiple myeloma, but the material studied by us differs significantly in electrophoretic and ultracentrifugal properties which clearly distinguish it from that of the above workers. Thus it appears that this type of Bence-Jones protein, distinguished by a solubility variation from the classical entity, may likewise be formed with a wide variation in physical properties. Future studies of this type of Bence-Jones protein are indicated.

The results of immunochemical studies to be reported in the subsequent paper indicate that the material is related antigenically to some of the normal serum globulins and to a Bence-Jones protein having the classical solubility properties.

SUMMARY

A Bence-Jones protein differing in solubility from the classical entity has been purified and characterized physically. Under certain conditions it shows electrical and molecular homogeneity. In monodisperse form it possesses a molecular weight near 31,000.

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IMMUNOCHEMICAL RELATION OF BENCE-JONES PROTEINS TO NORMAL SERUM PROTEINS*

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(Received for publication, January 17, 1955)

The Bence-Jones proteins have been reported to contain two immunological entities. However, in view of their varying physicochemical properties, it might be anticipated that they would show greater complexities in this respect. Although they are generally considered to be discrete substances and unrelated antigenically to the normal human serum proteins, the previous experimentation employed permits a reasonable doubt as to the validity of the conclusions reached. Thus this point appeared worthy of reinvestigation.

Collier and Jackson (1) have indicated that Bence-Jones proteins probably appear exclusively in the urine of all patients with multiple myeloma. The disease is usually concomitant with a hyperglobulinemia of one or more serum globulins, and the possibility presents itself that the Bence-Jones proteins may be fragments of these molecules. This was investigated by quantitative immunochemical studies of Bence-Jones proteins, γ-globulin fractions, and pooled normal human serum proteins. The results show that antisera to the two Bence-Jones proteins gave marked cross-reactions with normal serum proteins. This indicates a structural similarity and strongly suggests that the Bence-Jones proteins may indeed be fragments of certain normal serum protein molecules.

EXPERIMENTAL

Electrophoretic analyses were carried out in diethyl barbiturate buffer of 0.1 ionic strength, pH 8.6, at potential gradients near 5.5 volts per cm. The sedimentation analyses were performed in the Spinco apparatus at gravitational fields near 2.5×10^5 and employed a potassium phosphate buffer, 0.2 ionic strength, pH 7.4.

The normal γ_2- and γ_1-globulin fractions of human serum were analogous to those previously employed (2, 3). In addition to these, two Bence-Jones proteins, which will be designated BJ-LP and BJ-ZE, the γ_2-globu-

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lin of a multiple myeloma serum designated γ_2-KL, and normal human serum were employed as precipitinogens. The properties of BJ-LP have been described in the previous report (4). The BJ-ZE was prepared by a combination of salt and ethanol fractionation techniques. It was found to possess less than 0.03 per cent methionine when subjected to microbiological assay.[1] This means that the BJ-ZE protein has less than 0.05 mole

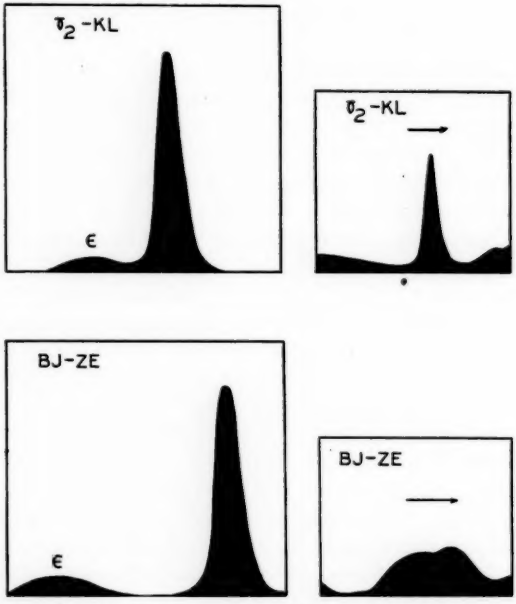

FIG. 1. Electrophoretic (left) and ultracentrifuge (right) diagrams of proteins γ_2-KL and BJ-ZE. Time of electrophoresis, γ_2-KL 209 minutes, BJ-ZE 200 minutes; time of ultracentrifugation, γ_2-KL 83 minutes, BJ-ZE 156 minutes.

of this amino acid per 35,000 gm. and may be considered to lack it. Results of electrophoretic and ultracentrifugal experiments on antigens BJ-ZE and γ_2-KL appear in Fig. 1.

BJ-ZE shows two components in the ultracentrifuge. The slower of these sediments with a velocity which suggests a molecular weight near 35,000. The faster component may be an aggregate of the first if the ultracentrifugal properties of this protein are analogous to those of BJ-LP in

[1] We wish to thank B. E. Kline for carrying out this analysis. The limits of the assay employed would not allow for the detection of less than 0.03 per cent methionine.

this respect (4). Its low solubility below pH 6 prevented experimentation in the range in which BJ-LP has been found to exist as a monomer.

Both the Bence-Jones proteins and the γ-globulins were sufficiently pure, and their physical properties were such that they could be stated to be free of contamination with each other. This obviates the consideration that the immunological cross-reactions experienced could be due to contamination.

Rabbit and chicken antisera were prepared against BJ-LP, BJ-ZE, and γ_2-KL by the adjuvant technique of Freund and McDermott (5). In some cases the γ-globulins of the rabbit antisera were separated by the method of Nichol and Deutsch (6). Quantitative precipitin reactions were carried out by methods previously described (7).

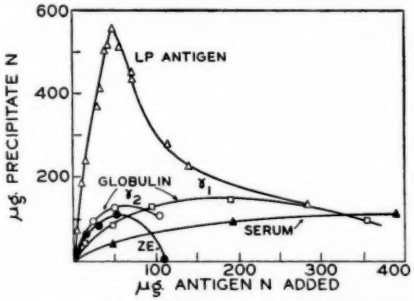

FIG. 2. The reactions of rabbit anti-BJ-LP serum with various precipitinogens

Results

The data will be considered with respect to the reactions of various precipitinogens with antiserum to BJ-LP, BJ-ZE, and γ_2-KL.

Reactions of BJ-LP Antibody—The results of the reaction of BJ-LP with its rabbit antiserum are presented in Fig. 2. This antigen stimulated good antibody production in rabbits to give a precipitin system which obeys the Heidelberger and Kendall (8) relationship. At the point of maximal precipitation the composition of the specific precipitate closely approximates Ab_2An when the rabbit antibody (Ab) is considered to have a molecular weight of 160,000 (9) and the BJ-LP antigen (An) to be 31,000 (4).

This antiserum also reacted strongly to normal γ_2- and γ_1-globulins, normal human serum, and BJ-ZE. γ_2-KL, however, failed to give any significant precipitate with a rabbit BJ-LP antibody preparation when studied over antigen additions from 5 to 353 γ of γ_2-KL antigen N. The latter amount gave only 15.4 γ of N precipitate, the amount at 118 γ of antigen N being 5.6 γ. The amount of specific precipitate given by normal human serum appears to be in proportion to its content of γ-globulins.

The precipitin reaction of rabbit anti-BJ-LP serum with γ-globulins is not due to the presence of Bence-Jones protein. Thus the addition of 90 γ of γ_2-globulin N to 1 ml. of BJ-LP rabbit antiserum yields an amount of specific precipitate equal to 15 γ of the homologous antigen. The γ_2-globulin employed would have had to contain near 17 per cent BJ-LP, an amount far in excess of that which could be readily detected both by electrophoretic and ultracentrifugal analysis.

The BJ-LP antiserum does not contain antibody directed against a γ-globulin impurity of the antigen. This is shown by the results of what will be termed *inhibition reactions*. These consist in determining the amount of specific precipitate given by BJ-LP in the presence of a second

TABLE I

Results of Blocking Reactions of Rabbit BJ-LP Antibody Reaction by Various Proteins

BJ-LP added	Blocking protein	Amount	Ppt.	Ppt. blocked
γ N		γ N	γ N	γ N
45.2		0	287	
0	γ_2-Globulin	115	112	
45.2	"	115	185	214
0	γ_2-KL	236	0	
45.2	"	236	144	193
0	γ_1-Globulin	213	7	
45.2	"	213	144	150
0	Human serum	1952	38	
45.2	" "	1952	139	186
0	BJ-ZE	292	0	
45.2	"	292	283	None

protein. The homologous antigen is employed at a level that will give maximal specific precipitation or be in the region of slight antigen excess. The simultaneous addition of a second antigen that combines with antibody produced against the homologous antigen will result in a marked decrease in specific precipitation because of conditions that simulate an excess of antigen. If a specific antibody to the second protein is present, the precipitate formed will be the sum of the individual reactions. Data of this type of experiment are presented in Table I and clearly show that all of the proteins tested block the homologous reaction. The γ_2-KL antigen gave no specific precipitation at any antigen level, but exerted a strong inhibiting effect. This indicates that the two proteins in question possess immunochemical similarities, even though they are vastly different in their ultracentrifugal and electrophoretic properties.

The reactions of chicken anti-BJ-LP serum under isotonic conditions and in 8 per cent NaCl are presented in Fig. 3. Goodman *et al.* (10) have

reported that far greater specific precipitation occurs for chicken antigen-antibody systems at higher salt levels. The marked differences previously reported do not obtain for the BJ-LP system, and the broad zone of maximal precipitation at the higher salt concentration is indicative of a decreased sensitivity. For this reason no further studies were carried out in 8 per cent NaCl.

Chicken anti-BJ-LP serum reacts weakly with normal human serum, and, in contrast to rabbit anti-BJ-LP serum, yields a small amount of precipitation with γ_2-KL. However, the data for blocking reactions with normal human serum and γ_2-KL, as presented in Fig. 3, reveal that these pro-

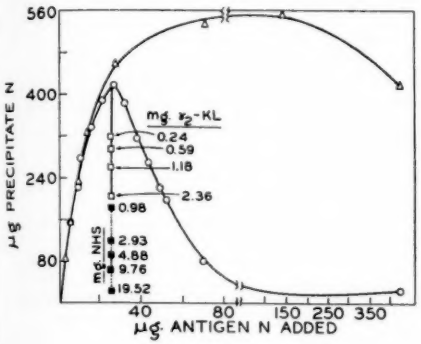

FIG. 3

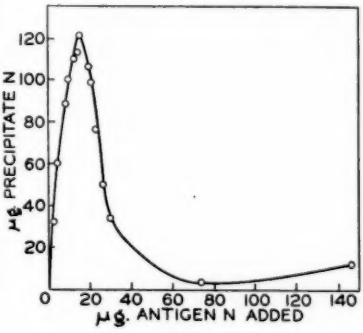

FIG. 4

FIG. 3. The reactions of chicken anti-BJ-LP serum with BJ-LP at 8 per cent NaCl (△) and at 0.15 M NaCl-borate buffer (○). The blocking reactions with γ_2-KL (□) and normal human serum (■) were carried out at a constant level of 23 γ of BJ-LP nitrogen.

FIG. 4. The reaction of BJ-ZE with its rabbit anti-γ-globulin.

teins very strongly block the homologous reaction, normal human serum being much more effective than γ_2-KL.

Antisera to BJ-ZE—BJ-ZE did not prove to be an effective antigen. A weak rabbit antiserum was obtained after prolonged immunization. The γ-globulin fraction was separated and reconstituted to twice its serum concentration to give a workable precipitin system. The results of the immunochemical reaction of this antibody preparation are presented in Fig. 4. The antibody also reacted with all of the other antigens tested. From the data of Table II it is apparent that the specific precipitation experienced with heterologous antigens is cross-reactive in nature. It is interesting that BJ-LP blocks the reaction of BJ-ZE with its antibody, whereas the reverse was not true (see Table I).

Once again the amount of specific precipitation given by heterologous

antigens was too great to be attributed to contamination with Bence-Jones protein. Furthermore, the results of the blocking reactions also failed to support this possibility.

Antisera to γ_2-KL—Both rabbit and chicken antibodies to this protein were utilized. The specific precipitin results for chicken antiserum (Fig.

TABLE II

Results of Blocking Reactions of Rabbit BJ-ZE Antibody Reaction by Various Proteins

BJ-ZE added	Blocking protein	Amount	Ppt.	Ppt. blocked
γ N		γ N	γ N	γ N
20.1		0	106	
0	γ_2-Globulin	115	0	
20.1	"	115	55	51
0	γ_2-KL	236	0	
20.1	"	236	75	31
0	γ_1-Globulin	213	18	
20.1	"	213	58	66
0	Human serum	4880	0	
20.1	" "	4880	56	40
0	BJ-LP	258	26	
20.1	"	258	71	61

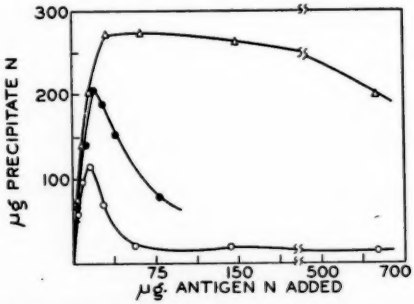

FIG. 5. The reactions of γ_2-KL with its chicken antiserum in 8 per cent NaCl (△) and under isotonic conditions (○). ●, the results of the precipitin reactions for the γ-globulin preparation of this serum.

5) reveal that the system is very sensitive to an increase in salt concentration, but the antigen excess region does not exhibit the usual decreased precipitation. Since the titer of the antiserum under isotonic conditions was somewhat low, the γ_2-globulin portion of the antiserum was separated and reconstituted to give a precipitin potency under isotonic conditions of the level shown in Fig. 5. This preparation was employed in further studies.

The two Bence-Jones proteins blocked the chicken antibody only weakly (Table III). It is interesting that BJ-ZE reacted strongly with the chicken γ_2-KL antibody at the level employed, but only a portion of this was blocking antibody. The proteins of normal human serum blocked the reaction completely. This was also found for rabbit anti-γ_2-KL globulin and will be reported more fully later. The rabbit antibody to γ_2-KL was only

TABLE III

Results of Blocking Reactions of Chicken γ_2-KL Antibody Reaction by Human Serum and Bence-Jones Proteins

γ_2-KL added	Blocking protein	Amount	Ppt.	Ppt. blocked
γ N		γ N	γ N	γ N
22.7		0	200	
0	Human serum	486	32	
22.7	" "	486	26	206
0	BJ-LP	258	5.6	
22.7	"	258	173	33
0	BJ-ZE	292	102	
22.7	"	292	238	64

TABLE IV

Results of Blocking Reactions of Rabbit γ_2-KL Antibody Reaction by Bence-Jones Proteins

γ_2-KL added	Blocking protein	Amount	Ppt.	Ppt. blocked
γ N		γ N	γ N	γ N
18.2		0	99.4	
0	BJ-LP	258	7.4	
18.2	"	258	91.5	15
0	BJ-ZE	292	6.3	
18.2	"	292	112	0 (+6)*

* Limit of assay method.

slightly blocked by BJ-LP, as shown by the data of Table IV. Thus the two Bence-Jones proteins block the homologous reaction of a γ_2-globulin with its chicken antibody only slightly and show little or no interference with the analogous rabbit precipitin system.

DISCUSSION

The two Bence-Jones proteins utilized in the present studies have uniquely different immunological properties. One of them, BJ-ZE, would appear to be similar to the proteins usually studied which are low or lacking in methionine (11–14). Immunologically it was similar to the Bence-

Jones proteins studied by Collier and Jackson (1) and by Moore, Kabat, and Gutman (15) in that it was a relatively poor antigen. In the present studies it is apparent from the results of blocking experiments that a portion of its antibody combines with serum globulins. Thus in such systems certain of the serum proteins behave as incomplete antigens by showing an ability to combine with antibody, even though they may fail to form specific precipitates. BJ-LP is a much better antigen than BJ-ZE, and it shows considerable cross-reaction with the serum globulins. A serum protein such as γ_2-KL, which fails to form a specific precipitate, does, however, combine with a portion of the antibody, as illustrated by the results of the blocking experiments. Such results indicate that the Bence-Jones proteins possess structural similarities to the serum globulins. It should be stressed that the difference in immunological response of rabbits and chickens to serum γ_2-globulins (3) is likewise reflected when Bence-Jones proteins are used as antigens.

A likely interpretation of the results presented in the present studies is that the Bence-Jones proteins are actually pieces (portions) of certain globulin molecules, as previously suggested by Rundles *et al.* (16). It is possible that in the accelerated synthesis of certain globulins in the condition of multiple myeloma a step in the normal synthetic mechanism may be limiting. Such limitation could lead to the formation of a fragment of the usual globulin molecule. The Bence-Jones proteins possess the electrophoretic mobility of the elevated serum globulins, and, when a Bence-Jones-positive myeloma patient exhibits a serum hyperglobulinemia characterized by two elevated globulin components, the urinary Bence-Jones protein likewise shows two components.[2] The specific fault involved in the formation of Bence-Jones proteins may be a failure to incorporate methionine adequately under certain conditions of accelerated globulin synthesis. Numerous studies have demonstrated the low level or absence of this amino acid in Bence-Jones protein (11–14), whereas the serum globulins of similar electrophoretic mobility always contain methionine (17, 18). Other workers have also shown that the total liver tumor proteins are characterized by a marked decrease in methionine compared to the normal tissue (19).

The fact that many different types of Bence-Jones proteins exist (4, 20-22) indicates that a possible metabolic failure or limitation with respect to methionine incorporation may involve a number of specific blood serum proteins. At times a number of proteins may be affected in such manner in a given individual.

The authors wish to acknowledge the technical assistance of Mr. George S. Steinmetz during a portion of this investigation.

[2] Kratochvil, C. H., and Deutsch, H. F., unpublished data.

SUMMARY

Highly purified Bence-Jones proteins have immunological similarities to those of normal serum globulins. This indicates structural similarities and suggests that they may be portions of normal serum protein molecules.

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PHOSPHORYLASE ACTIVITY OF SKELETAL MUSCLE EXTRACTS*

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(Received for publication, January 17, 1955)

Cori, Cori, and Green found that muscle phosphorylase exists in two forms, which they designated as phosphorylase *a* and phosphorylase *b* (1–5). Phosphorylase *a* exhibits 60 to 70 per cent of its maximal activity in the absence of AMP,[1] whereas phosphorylase *b* is inactive unless this nucleotide is present. Extracts of resting muscle were found to contain on the average 92 per cent phosphorylase *a*, but muscle stimulated to fatigue yielded mainly phosphorylase *b* (5). The hypothesis that the *a* form is converted to the *b* form *in vivo* during muscle contraction was advanced (5).

The present study was initiated to determine whether environmental temperature affected the phosphorylase content of skeletal muscle. Significant temperature effects were not noted; however, a surprising result was the finding that the muscle extracts contained mainly phosphorylase *b* rather than phosphorylase *a*. This was so, even though the animals were handled in ways to minimize muscle contraction. Experiments were conducted to rule out the possibility that a conversion of phosphorylase *a* to *b* occurred *in vitro* after extraction of the enzyme.

Methods

Animals—White female rabbits weighing from 2.5 to 4.5 kilos were used. No restrictions on rations[2] were imposed. Some of the rabbits were kept in a room[3] regulated at 5° and used at intervals as indicated. One rabbit was kept at 31°. The rabbits maintained at 24–28° were kept in the ordinary animal quarters, which were subject to small temperature variations.

Preparation of Muscle Extracts

Method 1—The procedure used was essentially the same as that described by Green and Cori in their method for the isolation of crystalline

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[1] AMP = adenosine-5′-phosphate; EDTA = ethylenediaminetetraacetate.

[2] Rabbit Breeder Paks, Albers Milling Company.

[3] The authors wish to thank Dr. Loren D. Carlson for making available the special animal quarters.

phosphorylase *a* (1, 6). Rabbits were anesthetized deeply by intravenous injection of Nembutal, care being taken to insure that no evident muscle contraction occurred during this process. The animals were bled from the jugular veins, and the back and hind leg muscles were excised immediately. During the excision, it was impossible to avoid some stimulation and a slight degree of muscle twitching was always present, despite the efforts made to keep these contractions minimal. The total lapse of time between injection of the anesthetic and completion of the removal of the muscles was between 10 and 15 minutes. The muscles were ground immediately in the cold room, extracted twice with cold distilled water,[4] and filtered through cheese-cloth. The extracts were centrifuged at 0° for 15 minutes at 2000 × *g* and filtered rapidly through a small wad of cotton. Activities of the extracts were determined immediately at this point unless noted otherwise.

Method 2—The rabbit was anesthetized with Nembutal, then bled, and the eviscerated carcass was cooled to 5° according to the procedure of Dounce *et al.* (7) for obtaining muscle in a manner that minimizes contractions. No twitching was observed during excision of the muscle. The excised muscle was treated as described in Method 1 for the preparation of the extract.

Method 3—The rabbit was anesthetized with Nembutal and then injected intraperitoneally with 900 mg. of $MgSO_4$ per kilo as described by DuBois *et al.* (8). The eviscerated carcass was chilled in ice before removal of the muscle. As in Method 2, no twitching was apparent during excision of the muscle. The remainder of the procedure is as described in Method 1.

Method 4—The procedure is the same as Method 1 except that neutral 0.001 M EDTA solution was used instead of water in extracting the ground muscle.

Method 5—The rabbits were injected intravenously with approximately 1 mg. of strychnine per kilo. Death occurred after severe convulsions of 1 to 3 minutes duration. The animals were bled from the jugular veins and handled as described in Method 1.

Preparation of Crystalline Phosphorylase a—The enzyme was prepared according to the procedure of Green and Cori (1, 6).

Phosphorylase Activity Determinations—Activity measurements on muscle extracts were made in the presence and absence of AMP at pH 6.0 according to the procedure of Illingworth and Cori (6). Preincubation of the enzyme in cysteine-glycerophosphate buffer was carried out for 8 minutes at 30° before the reaction was started. Preincubation of the enzyme

[4] Each extraction is made with an amount of water equal to the original weight of the ground muscle.

in the presence of glycogen (6) was found to be without effect on activity measurements in crude extracts and was not done routinely. Units of phosphorylase activity were corrected to pH 6.8, as has been described, and are given per ml. of original extract.

Results

Phosphorylase Activity and Temperature—Rabbits adapted to different environmental temperatures were used for a study of the total phosphorylase content[5] of muscle extracts. Table I gives the results obtained with animals kept at 5° and a control group maintained at 24–28°. The average total phosphorylase activity is approximately the same for the two groups. It was reported by Illingworth and Cori (6) that the phosphorylase content of muscle drops to low values when the room temperature is 30° or higher. A single rabbit, No. 24 of Table I, kept at 31°, did not show this, but a large series is obviously needed, since the individual variation in activity can be very great (1, 5).

Phosphorylase Activity and Rabbit Size—With the weight of muscle as an index of the size of the animals, the data of Table I show that there is an increase in total phosphorylase activity per ml. of extract with increasing rabbit size. This is equivalent to an increase of activity per unit weight of muscle, since the volume of extracting fluid always bears a constant ratio to the amount of tissue. The correlation between the two variables is significant with a probability of less than 2 per cent that the result was due to chance. If Rabbit 13 is excluded, this probability is less than 1 per cent.

Relative Amounts of Phosphorylase a and Phosphorylase b in Muscle Extracts—Most of the extracts from rabbits in either series of Table I showed low activities when tested in the absence of added AMP, indicating that the phosphorylase was predominantly in the *b* form. For example, the room temperature series (Rabbits 1 to 15) showed on the average only 32 per cent phosphorylase *a*, which is much lower than the 92 per cent reported by Cori (5) for a comparable series. The difference actually is more pronounced than these figures would indicate, since phosphorylase *a* present in the crude extracts gives a higher ratio ((activity without AMP)/(activity with AMP)) than the 0.65 used in the calculation of phosphorylase *a* concentration; this was pointed out by Cori (5) and was attributed to traces of AMP in the crude extracts. The average percentage of total phosphorylase in the *a* form was slightly lower (24 per cent) in the series at 5° of Table I, but this is not significant statistically.

Since evidence had been presented (5) that muscular contraction causes

[5] Activity determined in the presence of added AMP (1×10^{-3} M) is taken as total phosphorylase activity (6).

TABLE I

Phosphorylase Activity of Muscle Extracts

Extracts prepared by Method 1. Activity determinations carried out as described under "Methods." The figures in parentheses are the number of days the rabbits were kept at the temperature indicated. The weight of back and hind leg muscle obtained in each case is given in the second column. The temperatures at which the animals were kept are as follows: Rabbits 1 to 15, 24–28°; Rabbits 16 to 23, 5°; Rabbit 24, 31°.

Rabbit No.	Muscle	Activity of extract		Phosphorylase *a*
		+ AMP*	− AMP	
	gm.	*units per ml.*	*units per ml.*	*per cent*†
1	325	1260	130	16
2	330	770	380	76
3	340	1320	300	34
4	370	1270	360	44
5	390	1000	130	20
6	395	750	70	15
7	400	840	110	21
8	415	1100	100	14
9	480	1820	20	2
10	515	1470	1310	100‡
11	600	1200	1060	100‡
12	605	1690	310	28
13	650	760	20	4
14	650	2360	20	1
15	685	1420	70	5
Average.....		1270	293	32
16 (18)	200	1240	130	16
17 (21)	400	1040	0	0
18 (26)	410	1130	90	12
19 (60)	660	1180	230	19
20 (105)	670	1870	60	5
21 (54)	650	1680	130	12
22 (63)	600	1760	600	52
23 (11)	375	1070	550	79
Average.....		1370	220	24
24 (27)	465	1560	80	8

* Added in the activity assay system.

† Calculated on the basis that phosphorylase *a* is 65 per cent active without AMP (1).

‡ Activity without AMP > theoretical for phosphorylase *a*.

conversion of phosphorylase *a* to *b*, it appeared important to obtain muscle in as completely a resting condition as possible. Methods that have been shown to preserve muscle adenosine triphosphate (7, 8) were used; however, again phosphorylase was found mainly in the *b* form. In one experiment, extract prepared by Method 2 was found to contain 810 units per ml. of total phosphorylase,[4] of which all was phosphorylase *b*. In another experiment, extract prepared by Method 3 was found to contain 800 units per ml., of which 87 per cent was phosphorylase *b*.

Ruling Out Formation of Phosphorylase b in Vitro—Extracts obtained before or immediately after filtration through cheese-cloth were tested and found to contain the same amount of phosphorylase *b* as was present after the centrifugation step (see Method 1).

The possibility was considered that the concentration of PR enzyme[6] was unusually high in the crude extracts and that phosphorylase *a* was being converted to phosphorylase *b* in the handling of the extract or in the process of carrying out an activity test. Several types of evidence made this appear extremely unlikely: (1) When preincubation of the diluted extract in cysteine-glycerophosphate buffer was omitted entirely, essentially the same relative phosphorylase *b* to *a* ratios were obtained as with the preincubation, although slightly lower total phosphorylase activities were found. In these experiments fresh cold extract was diluted rapidly in cold buffer and immediately mixed with substrate for the activity test. (2) Crystalline phosphorylase *a* was added to the fresh crude extract, and its activity, as tested without added AMP, was completely recoverable under the usual conditions. (3) Fluoride was added to the fresh crude muscle extracts in a concentration of 0.1 M. This did not alter the relative amounts of phosphorylase *b* and *a* found. It has been shown that F^- at this concentration effectively inhibits the PR enzyme (9).

Extraction of Muscle with EDTA—It was found that muscle extracts containing only phosphorylase *b* could serve perfectly well for the isolation of crystalline phosphorylase *a* by the method of Green and Cori (1). For example, extracts of Rabbits 13, 14, and 17 of Table I were used for the isolation of crystalline phosphorylase *a*, which was obtained in good yields. These observations pointed to a conversion of phosphorylase *b* to *a in vitro*, which is the subject of the accompanying paper (10). It was determined that the reaction of phosphorylase *b* to *a* required a divalent metal ion; hence it appeared that extraction of muscle with EDTA solution (Method 4) rather than with water might give a more exact picture concerning the state of phosphorylase *in vivo*, since the effect of chance contamination by metals and a conversion of phosphorylase *b* to *a in vitro* would be avoided.

[6] The enzyme catalyzing a transformation of phosphorylase *a* to *b in vitro* is called PR enzyme (2, 4).

Table II presents the results obtained when the muscle from rabbits was divided into equal portions, one of which was extracted with water and the other with EDTA. It can be seen that in each case the EDTA extracts contained phosphorylase *b* almost exclusively. Separate experiments carried out with crystalline phosphorylase *a* and purified PR enzyme have shown no accelerating effect of added EDTA on PR enzyme action; thus these results cannot be interpreted as being due to an activation of PR enzyme. A larger series of animals is being studied by the EDTA extraction method.

TABLE II

Phosphorylase Activity of EDTA and Water Extracts of Rabbit Muscle

The muscle from each rabbit was divided into equal portions. Extractions by Method 1 (H_2O) and Method 4 (EDTA) were used.

Experiment No.	Activity of water extract		Activity of EDTA extract	
	+ AMP	− AMP	+ AMP	− AMP
	units per ml.	*units per ml.*	*units per ml.*	*units per ml.*
1*	1270	360	1150	60
2	1420	70	1065	10
3	1560	80	1540	40
4†	1850	190	1500	10
Average	1525	175	1314	30

* Experiment 1, Rabbit 4 of Table I; Experiment 2, Rabbit 15 of Table I; Experiment 3, Rabbit 24 of Table I.

† Rabbit killed with strychnine (Method 5).

DISCUSSION

It is difficult to determine with certainty the relative amounts of phosphorylase *a* and *b* present in muscle tissue in a given state of physiological activity, *i.e.* resting or fatigued muscle. According to the concept presented by Cori (5) and Sutherland (9, 11) the two forms of phosphorylase are interconvertible in the intact tissue, the particular balance existing at any time being determined by hormonal and other factors. In the type of assay employed in the present study, the muscle must first be removed from the anesthetized animal, ground, and extracted before any measurements are made. Complete control of the transformations that might tend to distort the ratio of phosphorylase *a* to phosphorylase *b* that existed *in vivo* cannot be obtained until the extraction step has been reached. In the extract itself phosphorylase *a* can change to phosphorylase *b* in the re-

action catalyzed by PR enzyme (2), but this reaction is relatively slow and ample time is available for enzyme assay before marked changes occur. A more rapid reaction is the conversion of phosphorylase *b* to phosphorylase *a* (10), which takes place if any of several common divalent cations are introduced into the extract. When the extraction of muscle is carried out with water, it is entirely possible that some conversion of phosphorylase *b* to *a* may occur before assays can be made; hence values such as those of Table I may indicate too much phosphorylase *a*. The use of EDTA would appear to be the most reliable method in approaching this problem.

The determination of whether resting skeletal muscle contains phosphorylase predominantly in the *a* or *b* form is of considerable physiological significance. Phosphorylase *b* has been interpreted as being relatively inactive *in vivo*, since it requires AMP in concentrations[7] that probably do not exist in muscle (5, 14). If resting muscle contains mainly phosphorylase *b*, as the present study would appear to indicate, then pronounced activation of the phosphorylase reaction under various conditions is possible. This is in keeping with Sutherland's concept that epinephrine increases glycogenolysis by causing a conversion of phosphorylase *b* to phosphorylase *a* (9, 11).

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SUMMARY

1. Muscle extracts of rabbits adapted to 5° showed the same total phosphorylase content as extracts from animals kept at 24–28°.

2. There was a significant correlation between the size of rabbits and the phosphorylase activity per given amount of muscle.

3. Extracts of resting muscle were found to contain phosphorylase predominantly in the form of phosphorylase *b*. Controls were conducted to rule out the possibility that phosphorylase *b* was formed through PR action during excision of the muscle or in the handling of the extracts.

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[7] The concentration of AMP required for half maximal activation of phosphorylase *b* is 5×10^{-5} M (12); however, the sensitivity of the enzyme to this nucleotide is increased in the presence of positively charged peptides such as protamine or synthetic polylysine (13).

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CONVERSION OF PHOSPHORYLASE *b* TO PHOSPHORYLASE *a* IN MUSCLE EXTRACTS*

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In 1943 rabbit skeletal muscle phosphorylase was isolated in the crystalline form by Green and Cori (1). The crystalline enzyme possessed 60 to 70 per cent of its maximal activity in the absence of AMP,[1] and in this characteristic differed from previously observed muscle phosphorylase that required AMP for activity. The type of phosphorylase represented by the new crystalline enzyme was designated as phosphorylase *a*, and phosphorylase with the absolute AMP requirement was designated as phosphorylase *b* (2). An enzyme (PR enzyme), which catalyzed a conversion of phosphorylase *a* to phosphorylase *b* *in vitro*, was discovered in muscle extract, and its action was studied extensively (2–4). Evidence was obtained that during muscle contraction phosphorylase *a* is changed to phosphorylase *b* and that during the recovery phase phosphorylase *a* is restored (5). Sutherland reported experiments showing phosphorylase *a* formation in the isolated muscle diaphragm (6).

In the preceding paper (7) it was found that phosphorylase as extracted from resting muscle is predominantly in the *b* form, *i.e.* requiring AMP. It has been determined that this enzyme can be converted readily to phosphorylase *a* in the cell-free extracts. The requirements for the reaction of phosphorylase $b \rightarrow a$ include a divalent metal ion and, under certain conditions, ATP.

Methods

The preparation of muscle extracts (Methods 1 to 5) and the determination of activity measurements and phosphorylase units are described in the preceding paper (7).

Reaction System for Conversion of Phosphorylase b to Phosphorylase a—1.0 ml. samples of muscle extract containing phosphorylase *b* are brought to 1.2 ml. final volume with water or various additions. After incubation

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[1] The abbreviations or contractions used in the paper include AMP, adenosine-5′-phosphate; ADP and ATP, adenosine di- and triphosphate; EDTA, ethylenediaminetetraacetate.

for specified intervals at 25°, samples are removed and diluted in 0.03 M cysteine-0.04 M glycerophosphate buffer, pH 6.0, and kept for 8 minutes at 30° prior to the activity test. The final dilution of the extract in the activity test system is 1:72.

Adsorption of Nucleotides with Norit—20 ml. of crude muscle extract were shaken with 1 gm. of Norit A (Pfanstiehl) for 5 minutes at 0° and the mixture was filtered through Whatman No. 42 paper.

Washing of Filter Paper—18 cm. Whatman No. 1 filters were washed with the following solutions added in succession: 50 ml. of 0.01 N HCl (per paper), 50 ml. of 0.01 N NaOH, 50 ml. of H_2O. The papers were allowed to dry at room temperature before use.

Filter Paper Extract—Twenty 18 cm. Whatman No. 1 filters were extracted with successive portions of 0.01 N HCl (total HCl = 1200 ml.). The extract was concentrated and dried *in vacuo*. 46.5 mg. of residue were dissolved in 10 ml. of H_2O and neutralized before use.

Ash of Filter Paper Extract—Half of the above extract before neutralization was dried in a platinum crucible (23.2 mg.) and ashed for 3 hours at 570°. The ash (17.6 mg.) was dissolved in 0.5 ml. of 1 N HCl and dried *in vacuo*. The residue (21.8 mg.) was dissolved in H_2O, adjusted to pH 7.0, and brought to 5.0 ml.

Results

Metal Requirement for Conversion of Phosphorylase b to Phosphorylase a—Fresh crude muscle extracts containing phosphorylase *b* were found to require only the addition of certain divalent cations and a short period of incubation to bring about complete conversion of the enzyme to phosphorylase *a*. Table I presents the data from a typical experiment in which the specificity of various metals for the reaction was determined. It can be seen that Ca^{++}, Sr^{++}, Ba^{++}, Zn^{++}, and Mn^{++} were all effective[2] in that the phosphorylase activity, as tested in the absence of added AMP, rose markedly. Metal ions, when added only to the test system, did not affect the relative concentrations of phosphorylases *a* and *b*. Total phosphorylase activity (measured with added AMP) also appears to be increased significantly in the transformation of phosphorylase $b \rightarrow a$; this finding will be discussed in a later section.

Nucleotide Requirements for Conversion of Phosphorylase b to a—In crude muscle extracts aged for 24 hours at 3° or kept in the frozen state for several days, phosphorylase *b* could still be converted to phosphorylase *a*, but it was found that under these conditions addition of ATP[3] was required.

[2] The slight effects of Fe^{++} and Co^{++} could not be repeated in other experiments.

[3] In most of the experiments reported in this paper, Pabst disodium adenosine triphosphate was used. In later experiments crystalline ATP from the same source was used and was found to give identical results.

Dialysis of muscle extracts or treatment with Norit also made the addition of ATP necessary for the conversion, as shown in Table II. Phosphorylase *b* is no longer converted to phosphorylase *a* by incubation with Mn^{++} alone, but the combination of Mn^{++} and ATP is effective.

TABLE I

Influence of Metal Ions on Conversion of Phosphorylase b to Phosphorylase a

1.0 ml. portions of muscle extract (Method 1) were brought to 1.2 ml. with water or additions and incubated for 20 minutes at 30°. 1×10^{-3} M ATP was present except in the first control. Activity measurements in the presence and absence of AMP were performed at a final 1:72 dilution of the extract. Units per ml. are calculated for undiluted extract in each case. Original activity of the extract before incubation, + AMP, 1300 units per ml.; − AMP, 20 units per ml.

Addition*	Activity		
	+ AMP	− AMP	$\frac{-\text{AMP}}{+\text{AMP}}$†
	units per ml.	*units per ml.*	*per cent*
Control (− ATP)	1260	70	6
" (+ ")	1400	180	13
K^+	1350	110	8
Mg^{++}	1320	120	9
Ca^{++}	1960	1730	88
Sr^{++}	2090	1840	88
Ba^{++}	1870	1230	65
Mn^{++}	2010	1840	92
Fe^{++}	1480	460	31
Co^{++}	1600	530	33
Ni^{++}	1710	80	5
Cu^{++}‡	1280	110	9
Zn^{++}‡	1710	1090	64
Hg^{++}‡	1430	140	10

* All salts were acetates or chlorides present at a final concentration of 1×10^{-3} M during the incubation.

† This quantity divided by 0.65 gives the percentage of phosphorylase *a* (1).

‡ 0.5×10^{-3} M during the incubation.

With the treated extracts (see above) ADP could be substituted for ATP with essentially identical results. AMP was also studied to determine whether it could serve to activate the conversion system of phosphorylase *b* → *a*; inconsistent behavior of this nucleotide was noted, depending upon the treatment of the extract. In frozen extracts no conversion occurred on incubation with 1×10^{-3} M AMP and Mn^{++}, although ATP (or ADP) at the same concentration was completely effective. With a 10-fold increase in the AMP concentration partial conversion took place. In Norit-treated extracts AMP was completely ineffective, which made it

appear unlikely that it could act as such in the reaction. Indeed, it was found that AMP could serve in Norit-treated extracts, if phosphocreatine was also added (Table III). As shown, phosphocreatine by itself was

TABLE II

Conversion System in Dialyzed or Norit-Treated Extracts

EDTA extract (No. I) from muscle (Method 4) was dialyzed for 3 hours at 5° against distilled H_2O and then incubated for 20 minutes at 25° with the additions indicated. Extract (No. II) obtained by Method 1 was treated with Norit and then incubated as above. Incubations with untreated control at 5°.

Extract	Ratio, phosphorylase activity (− AMP/+ AMP) after incubation with		
	No additions	3×10^{-3} M Mn^{++}	3×10^{-3} M Mn^{++} + 1×10^{-3} M ATP
	per cent	*per cent*	*per cent*
I. Before dialysis	9	86	86
" After "	8	2	72
II. Untreated	27	91	91
" Norit-treated	24	22	62

TABLE III

Ineffectiveness of AMP without Phosphocreatine

Muscle extract was obtained by Method 1 and frozen; it was thawed after 3 days and treated with Norit. Incubations carried out at 25°. 1×10^{-3} M Mn^{++} and 1×10^{-3} M Ca^{++} present in each incubation mixture. Phosphorylase activities carried out at 1:80 dilution of the incubation mixture.

Addition*	Ratio, phosphorylase activity (− AMP/+ AMP) after incubation for		
	0 min.	25 min.	120 min.
	per cent	*per cent*	*per cent*
None	10.4	9.7	13.7
Phosphocreatine	13.4	6.4	3.2
AMP	24.4†	31.7	33.8
Phosphocreatine + AMP	31.4†	75.5	91.8

* 1×10^{-3} M during incubation for each component.

† Norit-treated extracts contain almost no adenylic deaminase (8), and there is partial activation of phosphorylase *b* due to AMP carried over into the activity test system.

inactive. Assuming that a trace of ATP was still present in this extract, AMP could give rise to ATP in the presence of phosphocreatine through the myokinase and ATP-creatine transphosphorylase reactions.

Although it is difficult to determine the specificity of nucleotides in a crude system in which various transformations and interconversions may

occur, the following nucleotides have also been tested: inosine mono- and triphosphate, uridine mono- and triphosphate, cytidine mono- and triphosphate,[4] AMP (3′), guanosine-3′-phosphate, and cytidine-3′-phosphate. Of these, only uridine triphosphate showed a slight effect in activating the conversion reaction of phosphorylase $b \rightarrow a$; the possibility of traces of ATP in the uridine triphosphate has not been excluded.

Extent of Conversion in Different Muscle Extracts—When fresh extract is incubated at 25° with added metal ion, the increase in the − AMP/+ AMP phosphorylase activity ratio takes place very rapidly, sometimes reaching

TABLE IV

Extent of Conversion of Phosphorylase b to a in Rabbits under Various Conditions

The mean values are obtained by dividing the sum of the activity units per ml. of crude extract by the number of rabbits. Reaction system for conversion as described under "Methods;" the activities are determined after 30 minutes incubation at 25°.

Extract	No. of rabbits	Mean activities (units per ml.) after incubation with							
		No additions		10^{-3} M Mn^{++}		10^{-3} M ATP		10^{-3} M Mn^{++} + 10^{-3} M ATP	
		+ AMP	− AMP	+ AMP	− AMP	+ AMP	− AMP	+ AMP	− AMP
5°; Method 1.....	3	1680	345	1950	1500			2260	2190
24-28°; Method 1.	8	1570	130	1840	1470	1730	150	2060	1830
Strychnine anesthesia; Method 5.....	2	1620	70	1800	1500	1580	135	2080	1590
Special anesthesia; Methods 3, 4.....	2	1160	125	1380	1020	1250	250	1390	1100

a maximum within less than 5 minutes. With continued incubation after conversion is complete, the activities remain relatively stable for several hours; then a definite decline in the ratio occurs. After overnight incubation a phosphorylase *b* picture usually results.

The extent of conversion of phosphorylase $b \rightarrow a$ after approximately 30 minutes of incubation was studied with fifteen different muscle extracts with no additions and with added Mn^{++} plus ATP (Table IV). In most of the experiments incubation was also carried out with ATP alone (omitting Mn^{++}). The extracts used are separated into four groups (Table IV) according to the temperature at which the animals were kept or to the

[4] Pabst Laboratories, Milwaukee. We are indebted to Dr. F. M. Huennekens for providing the samples.

procedure used in killing the rabbits and preparing the extracts. Although they all contained phosphorylase predominantly in the *b* form originally, conversion to phosphorylase *a* occurred in each case, even with extracts obtained from strychninized rabbits.

It will be noted that, after conversion, the phosphorylase activity measured without AMP exceeds the maximum of 60 to 70 per cent found for crystalline phosphorylase *a* (1). It is unlikely that this can be accounted for on the basis of a carryover of AMP from the crude extract into the activity test reaction mixture. In an experiment in which the − AMP/+ AMP activity ratio was 100 per cent after conversion of phosphorylase *b* to *a*, the reaction mixture was treated with Norit and filtered. The filtrate still showed a ratio of 92 per cent, although absorption at 260 mμ indicated that no detectable amount of nucleotides remained. 5×10^{-6} M AMP would be easily detected in the absorption method, and this would be equivalent to less than 1×10^{-7} M AMP in the activity test.

Total phosphorylase activity is also increased significantly during the conversion of phosphorylase $b \rightarrow a$ (Table IV), as was confirmed by statistical analysis. Although no definite explanation can be given at this time for this phenomenon, several factors might be involved.[5] Activity measurements are carried out at pH 6.0, *i.e.* below the pH optimum for phosphorylase *a* or *b*.[6] The two enzymes may have different pH dependence curves, and it is possible that the metal ion or the nucleotide added for the conversion affects these curves differently. In crude extracts, deamination of AMP by adenylic deaminase proceeds at such a rate at pH 6.0 that the nucleotide added in the activity test is partially destroyed before the end of the reaction. This might result in values too low for phosphorylase *b*, whereas phosphorylase *a* activity would not be affected. This is consistent with the fact that addition of fluoride, which inhibits the deaminase, increases the total activity of phosphorylase before conversion but not after. Likewise, when a Norit-treated extract is used for the conversion (from which most of the deaminase has been removed), a smaller increase in total activity is observed. Finally, several preparations of crystalline phosphorylase *a* showed an increased activity when tested after a short incubation in the presence of divalent metal ions (Ca^{++}, Mn^{++}). A similar activation of the enzyme by these ions could occur in the crude extracts and thus contribute to the observed result.

[5] Cori and Cori have found that under certain conditions there occurred a 23 per cent *decrease* in total phosphorylase activity at pH 6.8 when phosphorylase *a* was converted to *b* by PR enzyme action (9).

[6] The pH optimum for phosphorylase is around 6.7 to 6.8, but, in crude extracts, activation is measured at pH 6.0 to minimize mutase action. Activity at pH 6.0 divided by 0.7 gives activity at pH 6.8 (10).

Properties of Conversion System—Although no definite proof has been obtained that the reaction of phosphorylase $b \rightarrow a$ is enzyme-catalyzed, it has been found that the presence of a protein fraction of the extract is necessary. If the pH of dialyzed muscle extract is lowered to pH 5.8, and the precipitate which forms[7] is removed, the phosphorylase *b* in the supernatant solution is no longer converted to phosphorylase *a* by incubation with Mn^{++} plus ATP. Addition of the fraction removed to the supernatant solution restores the reaction.

EDTA was found to block completely the reaction of phosphorylase $b \rightarrow a$, which was to be expected, since the system requires divalent metal ions. When calcium or manganous salts were added in molar excess over EDTA, the conversion system could be reactivated readily. Once phosphorylase *b* in the extracts had been converted to phosphorylase *a*, addition of EDTA had no effect on the phosphorylase activity itself.

Addition of F^- (0.04 M), arsenate (0.015 M), and cysteine (0.06 M) did not affect the degree of phosphorylase *b* to phosphorylase *a* conversion after 30 minutes of incubation of extract with Mn^{++} or Mn^{++} and ATP. In these experiments the extent of conversion at 30 minutes was essentially complete in all cases; hence slight effects on rate would not have been noticed.

Evidence of Phosphorylase a Formation during Conversion—It was important to establish beyond doubt that phosphorylase *a* was actually being formed and that the results observed were not due simply to AMP production and a carryover of this nucleotide into the activity test system. If this occurred, then naturally phosphorylase *b* could give the *a* picture. This possibility could be eliminated completely by several types of evidence.

If the total non-protein absorption of muscle extracts at 260 mμ is assumed to be due to AMP, the concentration of this nucleotide would be about 1.1×10^{-3} M in the extract. After dilution for determination of phosphorylase activity the concentration would be approximately 1.5×10^{-5} M AMP, *i.e.* a concentration capable of producing less than half maximal activation of phosphorylase *b* (11). Even in experiments in which adenine nucleotides were added to the conversion reaction mixture to concentrations of 1×10^{-3} M, the possible carryover of AMP would be too low to give half maximal activation.

The nucleotides present in the conversion reaction mixture after incuba-

[7] It is of interest that the fraction which is precipitated by this procedure contains the PR enzyme that catalyzes the reaction of phosphorylase $a \rightarrow b$ (2). Whether this means that the PR enzyme itself participates in the phosphorylase $b \rightarrow a$ process cannot be stated at this time, although it has been determined that purified PR enzyme alone does not catalyze the formation of phosphorylase *a* from phosphorylase *b* when incubated with Mn^{++} plus ATP.

tion, as carried out for phosphorylase *a* formation, do not include amounts of AMP detectable by the method of Kalckar (12). Even when AMP itself was added to the incubation mixtures in high concentrations (to 6×10^{-2} M), it had all disappeared within 15 minutes, owing to the action of adenylic deaminase.

Norit treatment of phosphorylase *a* after the conversion reaction removed all detectable traces of AMP, but did not alter the high ratio of − AMP/+ AMP activity.

Final proof of phosphorylase *a* formation resulted from its isolation in the crystalline form after conversion: To a crude muscle extract, prepared by Method 1 of the preceding paper (7), sufficient 0.5 M EDTA solution,[8] pH 7.0, was added to make the final concentration 0.001 M EDTA. One 450 ml. portion (Sample I) of this extract was carried through the regular steps in the preparation of phosphorylase *a* (1, 10), with the exception that the filtration step through paper was omitted. Dialysis prior to the removal of PR enzyme was carried out against distilled water.[9] Upon dialysis of the $(NH_4)_2SO_4$ precipitate, no protein separated from solution within 24 hours. Ultracentrifugal analysis on a similar fraction from a different preparation showed the presence of a single component with $s_{20} = 8.83$ in 0.03 M cysteine-0.04 M glycerophosphate buffer, pH 6.8. A second 450 ml. portion (Sample II) of the extract containing EDTA was treated with manganous acetate (final concentration of Mn^{++} in extract $= 3 \times 10^{-3}$ M) and ATP (final concentration $= 5 \times 10^{-4}$ M) and allowed to stand for 2 hours at 0° before proceeding with the preparation. The next steps were carried out as described above. With this portion a heavy precipitate of phosphorylase *a* developed, when the $(NH_4)_2SO_4$ precipitate was dialyzed. The enzyme was recrystallized twice in the usual manner. The microscopic appearance of the crystals resembled that shown for phosphorylase *a* (1). Table V shows the recoveries in the different steps of the preparation. A considerable loss of phosphorylase occurred in the isoelectric precipitation of PR enzyme, but this was compensated for by dissolving the 41 per cent saturated $(NH_4)_2SO_4$ fraction in only a small volume of water.

The twice recrystallized enzyme was contaminated with traces of PR enzyme, which made ultracentrifugal analysis difficult; however, the major component found (60 per cent of the total) had an s_{20} of 13.27 which is in

[8] The EDTA was added so that the control portion of the extract (Sample I) could be carried through the preparation without risk of conversion to phosphorylase *a* by chance contamination with metals. Dialysis was carried out against distilled water rather than tap water (1) for the same reasons.

[9] In 1945 Cori noted an apparent increase in the concentration of phosphorylase *a* following dialysis of frog and rabbit muscle extracts (5).

close agreement with the known value for phosphorylase *a* ($s_{20,w}$ = 13.2) (4). A second component with an s_{20} of 8.47 comprised 38.2 per cent of the total; this was presumably phosphorylase *b* ($s_{20,w}$ = 8.2) (4).

TABLE V

Phosphorylase Purification with and without Conversion to Phosphorylase a

Original extract obtained by Method 1 and EDTA added to final concentration of 0.001 M. Extract divided into two portions: Sample I, no conversion procedure; Sample II, converted to phosphorylase *a* as described in the text.

Step	Sample I				Sample II			
	Volume	+ AMP	− AMP	Total units	Volume	+ AMP	− AMP	Total units
	ml.	*units per ml.*	*units per ml.*	× 10^{-3}	*ml.*	*units per ml.*	*units per ml.*	× 10^{-3}
Original extract (with EDTA)	450	2,360	20	1062	450	2,360	20	1062
After incubation with Mn^{++} + ATP					450	2,450	2,350	1102
After dialysis	460	1,990	200	916	465	2,000	1,750	930
" removal of isoelectric ppt.	460	700	0	324	462	1,020	800	471
After dialysis of $(NH_4)_2SO_4$ ppt.	6.0	36,000	100	216	6.4	52,000	33,500	332

TABLE VI

Filter Paper Effects on Muscle Extract

Phosphorylase activities were determined on portions of muscle extract obtained by Method 1 and treated as indicated. Original activity of muscle extract before any treatment, + AMP, 1800 units per ml.; − AMP, 20 units per ml.

Treatment	Activity		
	+ AMP	− AMP	$\frac{-\text{AMP}}{+\text{AMP}}$
	units per ml.	*units per ml.*	*per cent*
Filtration through unwashed paper	2200	1700	77
" " washed paper	1740	20	2
Incubation with filter paper extract	2660	1420	53
" " " " ash	2340	1470	63

Effect of Filtration of Muscle Extracts—In the preceding paper (7) it was noted that rabbit muscle extracts containing only phosphorylase *b* could serve, nevertheless, for the isolation of crystalline phosphorylase *a*, when the method of Green and Cori (1) was followed. It was found that the *a* form of the enzyme appeared following filtration of the extract through

Whatman No. 1 paper. If the filtration step was omitted, partial conversion of the original phosphorylase *b* was found to occur during dialysis against cold tap water, but not during dialysis against distilled water. As presented in Table VI the effect of filtration can be accounted for by extraction of metals present in the paper. Washing of the paper abolishes the effect. When filtration is omitted and filter paper extract or ash is added, phosphorylase *a* is formed after a short incubation period. Incubation with no additions does not change the original activity.

Kritskiĭ and Kuvaeva (13), using a procedure based largely on that of Green and Cori (1) but in which filtration through paper and dialysis were avoided, claimed to have obtained a crystalline material showing a phosphorylase *b* type of activity.

Cori found that muscle extracts from rabbits killed with strychnine would not serve for the preparation of crystalline phosphorylase *a*. In the present study it has been found that the conversion of phosphorylase *b* → *a* does occur in this type of extract when divalent metal ions are added immediately (see Table IV); however, the pH of these extracts (pH 6.0 to 6.1) is lower than those obtained with barbiturate anesthesia and drops rapidly on standing to a point (pH 5.8) at which no conversion occurs with metal alone. When filtration or dialysis serves as the sole source of metal ions, it is possible that they may be introduced too late to effect a conversion.

DISCUSSION

In this study, evidence has been presented showing that the conversion of phosphorylase *b* to *a*, as it occurs in cell-free muscle extracts, requires a nucleotide containing high energy phosphate, in addition to a divalent metal ion.[10] Whether this implies that during conversion there is a direct phosphorylation of the enzyme or the formation of an "active" intermediate cannot be stated at this time. It is also possible that the function of ATP is concerned with the synthesis of a prosthetic group.

No definite information is available as to whether phosphorylase *b* present in crude muscle extract (7) is identical with the phosphorylase *b* produced from crystalline phosphorylase *a* with purified PR enzyme. Keller and Cori (4) have found that in this process a halving of the molecular weight

[10] Rosenfeld and Petrova (14) have reported a conversion of phosphorylase *b* to *a* in muscle extracts that were autolyzed for 48 hours at room temperature, stored for several weeks in the refrigerator, and finally alkalinized. It is very difficult to interpret what may have happened under their conditions. In the present study, no spontaneous conversion has been noticed during storage of the extracts. However, on a few occasions fresh crude extracts showed a slight increase in phosphorylase *a* when made alkaline.

of phosphorylase *a* occurs. Recent experiments that will be reported in detail in a subsequent publication show, however, that phosphorylase *b*, obtained by the action of PR enzyme on crystalline phosphorylase *a*, is also readily converted back to phosphorylase *a* in the conversion system described in this paper.

It is of interest to recall that the dimerization of another muscle protein, that of G-actin to F-actin (15), also requires a divalent metal ion and ATP. In this system the monomer units are thought to be linked together through the nucleotide prosthetic group and the metal. With phosphorylase the possibility of two monomer (phosphorylase *b*) units being linked together through a metal ATP complex appears to be unlikely, since very little adenine is found in crystalline phosphorylase *a* (16). Tracer techniques are being used in order to determine whether the metal or any portion of ATP is incorporated during the conversion of phosphorylase *b* to *a* in a purified system. Sutherland and Cori (6, 17) have presented evidence that epinephrine exerts its glycogenolytic effect by causing an increased concentration of active phosphorylase in tissues. This increase is due presumably to the conversion of phosphorylase *b* to *a*. Since the requirements for this reaction have now been established, a study of the influence of epinephrine on the mobilization of the various components involved may give a clue to the mode of action of this hormone.

The authors wish to thank Mr. Roger Wade for carrying out the ultracentrifugal analyses and Mrs. Jeanne Dills and Mr. Charles Beaudreau for their technical assistance.

SUMMARY

The conversion of phosphorylase *b* to phosphorylase *a* has been accomplished in cell-free muscle extracts.

Requirements for the reaction include a protein fraction of the extract, divalent metal ions, and ATP.

Crystalline phosphorylase *a* can be obtained from phosphorylase *b* after conversion of phosphorylase *b* → *a in vitro*.

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THE CONVERSION OF INACTIVE PHOSPHORYLASE TO PHOSPHORYLASE *b* AND PHOSPHORYLASE *a* IN LOBSTER MUSCLE EXTRACT

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In an investigation of phosphorylase activity in lobster muscle extract it was noted that there occurred during storage of the extract in the cold a several fold gain in total activity. Total activity is defined as the sum of activities of phosphorylase *a* plus phosphorylase *b*, *i.e.* activity in the presence of added adenosine-5′-phosphate (AMP). Phosphorylase *b*, in contrast to phosphorylase *a*, is inactive in the absence of AMP. Analysis of this phenomenon indicated that the following changes occurred: inactive phosphorylase → phosphorylase *b* → phosphorylase *a*. Temperature, AMP, fluoride, and a number of detergents were found to accelerate this transformation. Versene (ethylenediaminetetraacetate) was inhibitory.

EXPERIMENTAL

Tail muscles were obtained from "active" lobsters which were kept in an aerated tank at 10° filled with water containing salts of a composition similar to that of sea water.[1] The muscles were washed with tap water, passed through a cold meat grinder, mixed with an equal weight of cold distilled water, extracted for 10 minutes, and centrifuged. The muscle residue was reextracted as above, and the two supernatant fluids were combined and filtered.

Phosphorylase *a* activity was measured at 30° in a reaction mixture containing suitably diluted extract (generally 1:10 to 1:20), 0.015 M glucose-1-phosphate, 1 per cent glycogen, and 0.1 M sodium fluoride at pH 6.8. The fluoride was added in order to inhibit phosphoglucomutase and adenylic deaminase. Separate tests showed that the former enzyme was completely inhibited, while the latter was inhibited about 60 per cent by the

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[1] Lobsters which had become inactive because of storage in the cold out of water proved unsatisfactory for this work.

above concentration of fluoride.[2] Cysteine was omitted from the reaction mixture since it did not increase the activity of lobster muscle phosphorylase. For the determination of total phosphorylase, the assay mixture also contained 1×10^{-3} M AMP. Phosphorylase *b* was obtained as the difference between assays with and without AMP. The phosphorylase *a* of lobster muscle, in contrast to that of rabbit, did not appear to be stimulated by AMP. Satisfactory first order kinetics were observed, and all activities were expressed in phosphorylase units as previously defined (1).

Uridine-5′-phosphate and cytidine-5′-phosphate were kindly supplied by Dr. Gerhard Schmidt. UDPG (uridine diphosphoglucose) was a gift of Dr. Luis Leloir. Atlas Powder Company supplied Tween 80 (polyoxyethylene sorbitan monooleate). Aerosol OT (dioctyl sodium sulfosuccinate) was obtained from Eimer and Amend and CDEA (cetyldimethylethylammonium bromide) from the Eastman Kodak Company.

Results

In Fig. 1 is shown an experiment in which the total phosphorylase activity increased nearly 6-fold during incubation at 20° for 7.5 hours. The gain was mainly in phosphorylase *a*. Phosphorylase *b*, after an initial rise, reached a plateau and then declined. Little change occurred during the first 2 hours of incubation. Induction periods of varying duration have been observed in other experiments. The same extract, while being stored at 2° under toluene, underwent these changes at a slower rate and reached about the same end-point in 24 hours as did the extract at 20° in 7.5 hours[3] (Fig. 2). Dialysis did not affect the gain in total phosphorylase, but, as shown in Fig. 2 for the 24 hour period, the gain in the dialyzed extract was mainly due to phosphorylase *b*, while the non-dialyzed extract showed the usual gain in phosphorylase *a*. This suggested that dialysis removed a cofactor which is necessary for the phosphorylase *b* → phosphorylase *a* transformation, while the transformation, inactive precursor → phosphorylase *b*, could still occur in the dialyzed extract. An alternative

[2] Lobster muscle phosphorylase requires addition of glycogen for activity. When glycogen was omitted from the reaction mixture, no inorganic P was released and glucose-1-phosphate (determined as easily hydrolyzable P) remained unchanged. This indicates that phosphoglucomutase was blocked effectively by 0.1 M NaF and that there were no phosphatases present which could split glucose-1-phosphate. Spectrophotometric examination of the phosphorylase assay mixture, which contained 0.1 M NaF, indicated little or no conversion of added AMP to inosinic acid during the assay period.

[3] This meant that a given extract could be used for these experiments for a period of only 24 hours, and for technical reasons many experiments were carried out at the low temperature. Freezing the extract proved unsatisfactory, since activity was not preserved unchanged.

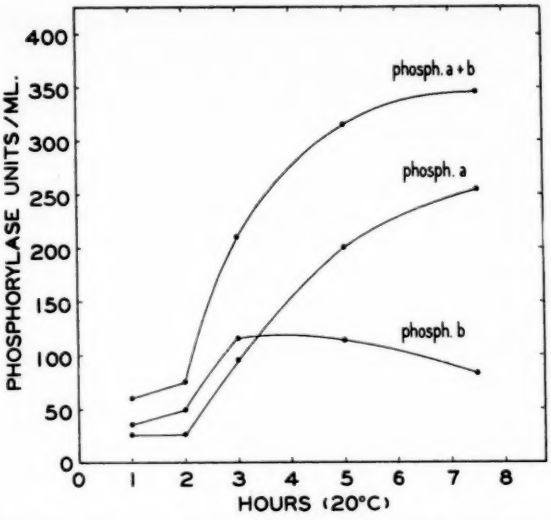

FIG. 1. A freshly prepared lobster muscle extract was kept at 20°, and aliquots were assayed at the times shown.

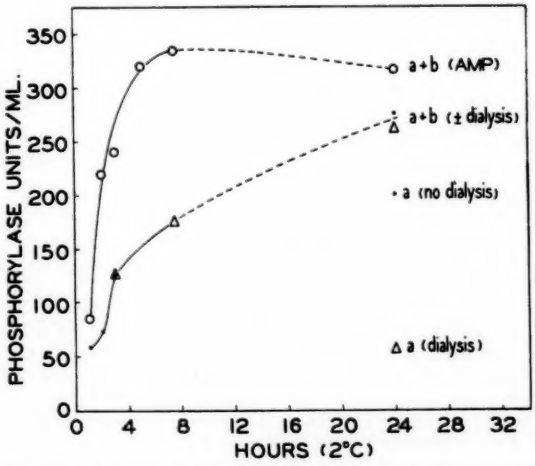

FIG. 2. The same extract as in Fig. 1 was kept at 2° under toluene. ○, phosphorylase $a + b$ in extract to which adenylic acid (6×10^{-4} M) was added; △, phosphorylase $a + b$ and phosphorylase a (after 24 hours) in extract dialyzed against 0.1 M sodium fluoride; ●, the same, but extract not dialyzed.

explanation was that the increase in phosphorylase *a* activity in the non-dialyzed extract was an apparent one and was simulated by the liberation of some nucleotide during incubation which increased the activity of phosphorylase *b*.[4] Purification of phosphorylase *a* from such extracts (see below) makes this explanation unlikely.

Fig. 2 shows that the addition of AMP, although rapidly destroyed by adenylic deaminase, has a marked accelerating effect. A similar effect could be produced by the addition of deproteinized muscle extracts of inactive lobsters (see below), but so far the substance which causes this effect has not been identified.

TABLE I

Changes in Phosphorylase Activity in Lobster Muscle Extract

Aliquots of a freshly prepared extract were kept under toluene and were treated as follows: Extract 1, kept at 2°; Extract 2, kept at 30° for 5 hours, then at 2°; Extract 3, kept at 2° with 2×10^{-6} M adenylic acid and 0.1 M NaF; Extract 4, kept at 2° with 2×10^{-4} M CDEA, a cationic detergent. At stated times tests were carried out for phosphorylase *a* and *b* activity. The values are given in units per ml. of extract.

Time of incubation	Extract 1		Extract 2		Extract 3		Extract 4	
	Phosphorylase *b*	Phosphorylase *a*	Phosphorylase *b*	Phosphorylase *a*	Phosphorylase *b*	Phosphorylase *a*	Phosphorylase *b*	Phosphorylase *a*
hrs.								
0	210	105	210	105	210	105	210	105
2			365	165			250	415
4	380	70	245	370	280	195	75	540
24	300	80	20	690	0	580	100	660
48	55	685	0	685	0	645	70	615

In Table I is shown the accelerating effect of temperature on the transformation. Phosphorylase *b* rises first, both at 2° and 30°, and later declines, the decline being accompanied by a rise of phosphorylase *a*. When AMP plus fluoride[5] was added, the latter in order to slow down the action of adenylic deaminase, the over-all reaction was accelerated and phosphorylase *b* disappeared completely; *i.e.*, the same activity was obtained in the phosphorylase test whether or not AMP was added. (The amount

[4] Phosphorylase *b* of lobster muscle was found to be half saturated with about 1×10^{-6} M AMP. Inosine-5′-phosphate, uridine-5′-phosphate, and cytidine-5′-phosphate, when added to the test system, increased phosphorylase *b* activity about one-half as much as did AMP. These three nucleotides, as well as 2×10^{-4} M UDPG, failed to activate purified phosphorylase *b* of rabbit muscle.

[5] In several experiments, addition of fluoride alone had some accelerating effect on the transformation.

of AMP added to this extract was diluted sufficiently in the activity test to have no discernible effect.) In the presence of the cationic detergent[6] CDEA the gain in phosphorylase *a* was faster at 2° than in the absence of detergent at 30°. In this and in other experiments the ultimate level of phosphorylase activity which was reached was independent of the addition of accelerators.

The pH changes during storage of extracts at 2° were small; a drop from an initial level of pH 7.2 to 7.0 occurred during 24 hours while the changes in phosphorylase activity took place (Table II). The pH optimum for activity of lobster muscle phosphorylase was found to be at 6.8.

In Table III an experiment is recorded in which phosphorylase formed from the inactive precursor in the presence of detergent was subjected to a 20-fold purification. The alcohol fractionation step should separate the

TABLE II

Changes in pH of Crude Lobster Muscle Extract during Storage at 2°

Hrs.	pH	Phosphorylase		
		b	*a*	*a* + *b*
		units per ml.	*units per ml.*	*units per ml.*
0.5	7.22	80	80	160
3.5	7.08	120	70	190
7	6.90	75	90	165
24	7.02	30	300	330

enzyme from any free nucleotides. The purified material contained mainly phosphorylase *a* and represented a 3-fold gain in the amount of phosphorylase *a* over that found in the original extract.

Further evidence that the material formed is phosphorylase *a* was obtained in the following experiments performed by N. B. Madsen.[7] To a lobster muscle extract (which has reached an end-point of its own phosphorylase transformations) was added rabbit muscle phosphorylase *b*, prepared by the action of PR enzyme (2) on crystalline rabbit muscle phosphorylase *a*, and 0.2 M sodium fluoride. Initially there were present,

[6] 1×10^{-3} M Aerosol OT, an anionic detergent, also had an accelerating effect on the transformation, but the total phosphorylase activity decreased with time. Tween 80, a non-ionic detergent, had a weaker effect. It should be mentioned that the above cationic and anionic detergents, but not the non-ionic detergent, are inhibitory in the phosphorylase test in concentrations above 5×10^{-5} M. The effect of these surface-active agents on phosphorylase activity will be reported in a separate paper.

[7] The authors are indebted to Mr. N. B. Madsen for making these data available to them.

per ml., 1900 units (not quite 1 mg.) of rabbit muscle phosphorylase *b*. After 60 minutes of incubation at 30°, the gain in phosphorylase *a* was 990 units, and after 120 minutes, 1100 units, at which time phosphorylase *b* had decreased to 560 units. Similar results were obtained with another lobster muscle extract; of 1200 units of phosphorylase *b* added per ml. of extract, 760 units were converted to phosphorylase *a* during 120 minutes of incubation at 30°. In each case the extract, when incubated without addition of phosphorylase *b*, showed insignificant changes in phosphorylase activity. The system responsible for the conversion of phosphorylase *b* to phosphorylase *a* proved to be quite labile, since aging or repeated freezing and thawing of the extract destroyed its activity.

TABLE III

Purification of Lobster Muscle Phosphorylase

Treatment	Phosphorylase				Specific activity
	b	*a*	*a* + *b*	$\frac{a}{a+b}$	
	units $\times 10^{-3}$	*units* $\times 10^{-3}$	*units* $\times 10^{-3}$	*per cent*	*units per mg. protein*
Extract from 240 gm. tail muscle	70	39	109	36	12.5
Stored with 5×10^{-4} M CDEA overnight at 0° and centrifuged	26	212	238	89	79
Polyethylene glycol* added to 60%, cooled to −30°; ethanol added to 30%. Ppt. dissolved in 0.03 M imidazole, pH 6.8, for test	28	126	154	82	252

* The polyethylene glycol (average mol. wt. 600) caused a small precipitate which was removed by centrifugation and contained 8 per cent of the phosphorylase.

The following additional observations about lobster muscle phosphorylase may be mentioned. The rise in activity on storage of the muscle extract appeared to be dependent on the physiological condition of the lobster. Fourteen lobsters, swimming actively in a tank, yielded extracts of initially low phosphorylase content, and, as shown in Tables I to III, there was a considerable amount of phosphorylase *b* present. By contrast, eight out of nine "dormant" lobsters, kept out of water in cold storage, yielded extracts of initially high phosphorylase levels with a predominance of the phosphorylase *a* form. Except in one case in which there was a 1.5-fold rise, these extracts did not undergo a change in phosphorylase content, while in the extracts from "active" lobsters the phosphorylase content increased, on an average, 3.5 times, reaching about the same level as was found initially in the extracts of "dormant" lobsters. Another dif-

ference noted was the much higher level of a substance in deproteinized muscle extracts of dormant as compared to active lobsters which accelerated the phosphorylase transformations

It was found that the inactive form of phosphorylase is not bound to particles which are sedimented in 45 minutes by centrifugation at 20,000 × *g* (Table IV).

In one experiment with a frog muscle extract, kept at 2°, the total phosphorylase content rose 1.8 and the phosphorylase *a* content 1.6 times in 24 hours. In extracts of rabbit and rat muscles, the changes in phosphorylase content, if they occurred at all, were small in comparison. In one rabbit muscle extract, phosphorylase *a* rose from 445 to 600 units per ml.,

TABLE IV

Failure to Concentrate Inactive Phosphorylase of Crude Lobster Muscle Extract by Centrifugation

Centrifugation was at 20,000 × *g* for 45 minutes.

Extract	Period of storage at 2°	Phosphorylase *a* activity
	hrs.	*units per ml.*
Original extract, stored without centrifugation at 2°	0	180
	3.5	355
	48	1060
Top half of centrifuged extract withdrawn and stored at 2°	3.5	270
	48	805
Bottom half of centrifuged extract mixed and stored at 2°	3.5	285
	48	945

while total phosphorylase rose from 700 to 1090 units per ml. during storage for 4 hours at 2°. The addition of AMP or of 0.1 per cent Tween 80 caused only a slightly greater increase.

DISCUSSION

The occurrence of an inactive form of phosphorylase in lobster muscle which depends on the state of activity of the animal is of interest in relation to the following observations. An inactive form of the enzyme which is converted to an active form under the influence of epinephrine has previously been described in mammalian liver (3). Sutherland (4) also observed a rapid conversion of phosphorylase *b* to phosphorylase *a* when epinephrine was added to isolated respiring rat diaphragm; there was, however, no change in total phosphorylase content.

The reversible interconversion of these forms is presumably catalyzed by special enzymes and plays a physiological rôle. The action of PR

enzyme (5) and of trypsin (6) on phosphorylase *a* has been shown to consist in a halving of the molecular weight of phosphorylase *a*. The PR enzyme is completely inhibited by 0.1 M fluoride (7), and the fact shown in Table I and in other experiments that this concentration of fluoride does not prevent the transformation of phosphorylase *b* to phosphorylase *a* suggests that this transformation is catalyzed by a different enzyme.

No information is available at the present time about the molecular weight of the inactive form of phosphorylase. By means of dialysis it has been possible to separate the conversion of this form into phosphorylase *a* into two steps, the first of which consists in the formation of phosphorylase *b*. This step is completely blocked by the addition of 3×10^{-3} M Versene. The effect of Versene on the phosphorylase *b* → phosphorylase *a* step has not been tried and the possible participation of a heavy metal in these transformations has not been tested.

In a paper which Fischer and Krebs (8) were kind enough to send us before publication, a transformation of phosphorylase *b* to phosphorylase *a* in rabbit muscle extract is demonstrated which requires a divalent metal ion and a nucleotide as cofactors.

SUMMARY

Extracts prepared from the tail muscle of lobsters maintained in a tank contain an inactive form of phosphorylase which is converted to phosphorylase *b* and then to phosphorylase *a* during incubation of the extract. The average increase in phosphorylase activity in fourteen extracts was 3.5-fold. Temperature, adenosine-5′-phosphate, fluoride, and a number of detergents were found to accelerate this transformation. Versene inhibited the reaction completely. Addition of rabbit muscle phosphorylase *b* to these extracts results in its conversion to phosphorylase *a*. Extracts prepared from muscle of dormant lobsters kept out of water under refrigeration showed initially a higher phosphorylase content and no rise on incubation. A frog muscle extract showed a nearly 2-fold rise in phosphorylase activity during incubation, while extracts from rat and rabbit muscle showed only small changes. The significance of these findings is discussed in the light of previous observations.

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PURIFICATION AND PROPERTIES OF THE PHOSPHORYLATING ENZYME FROM SPINACH*

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The preparation of a soluble system from pig heart capable of coupling the oxidative decarboxylation of α-ketoglutarate with the esterification of inorganic phosphate was first described in 1951 (1). The enzyme responsible for the phosphorylation, referred to as the phosphorylating enzyme (P enzyme), was separated from the α-ketoglutaric dehydrogenase and succinyl CoA[1] deacylase and extensively purified (2). It has been shown that the over-all reaction catalyzed by the P enzyme is

(1) $$\text{Succinyl-S-CoA} + \text{ADP} + \text{P} \xrightleftharpoons{(Mg^{++})} \text{succinate} + \text{HS-CoA} + \text{ATP}$$

These results were confirmed by Hift *et al.* (3). More recently (4, 5) it has been found that the nucleotide involved in the primary reaction with succinyl CoA, as catalyzed by the heart P enzyme, is GDP (or IDP) and that ATP is formed only through a secondary transphosphorylation catalyzed by a separate enzyme. The reaction sequence has been formulated as follows (5):

(2) $$\text{Succinyl-S-CoA} + \text{GDP} + \text{P} \rightleftharpoons \text{succinate} + \text{HS-CoA} + \text{GTP}$$

(3) $$\text{GTP} + \text{ADP} \rightleftharpoons \text{GDP} + \text{ATP}$$

It can be seen that Reaction 1 is the sum of Reactions 2 and 3.

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[1] The following abbreviations are used: CoA or CoA-SH, coenzyme A (reduced); succinyl CoA or succinyl-S-CoA, succinyl coenzyme A; ATP, GTP, and ITP, adenosine, guanosine, and inosine triphosphate; ADP, GDP, and IDP, adenosine, guanosine, and inosine diphosphate; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide; P, orthophosphate; DNP, 2,4-dinitrophenol; Tris, tris(hydroxymethyl)aminomethane.

The P enzyme has now been purified from spinach leaves. In contrast to the preparations from heart (2), the spinach enzyme appears to be completely free of myokinase. Freedom from this enzymatic contaminant has made it possible to determine the equilibrium constant for the reaction and to carry out certain isotopic tracer experiments which might throw some light on the reaction mechanism (6, 7). Although the enzyme prepared from spinach is similar in many respects to the heart preparation, there are some important differences. Perhaps the most interesting of these is that neither GDP nor IDP can substitute for ADP in the reaction catalyzed by the spinach enzyme. However, the possibility exists that

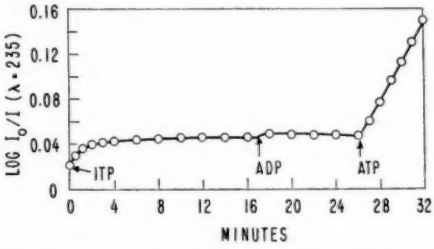

FIG. 1. Nucleotide specificity of spinach P enzyme. The system contained 100 μmoles of Tris buffer, pH 7.4, 10 μmoles of $MgCl_2$, 100 μmoles of succinate, 0.28 μmole of CoA, 8.3 μmoles of L-cysteine, 0.25 μmole of nucleotide, added as indicated by the arrows, and 0.01 mg. of purified P enzyme (specific activity 35). Final volume made up with water to 3.0 ml. Silica cells, d = 1.0 cm.; temperature, 25°. CoA omitted from blank cell and replaced by an (optically) equivalent amount of AMP. Reaction followed through the increase in absorption of light at wave-length 235 mμ due to the thio ester bond of succinyl CoA.

GDP, IDP, or another nucleoside diphosphate, is a firmly bound prosthetic group of this enzyme.

Isolation of Enzyme

Assay—The enzyme was assayed as in previous work (2) by following the rate of hydroxamic acid formation from succinate, ATP, and catalytic amounts of CoA in the presence of hydroxylamine. 1 unit is defined as the amount of enzyme catalyzing the formation of 1.0 μmole of hydroxamic acid in 30 minutes. Occasionally, a spectrophotometric assay has been used, based on the ultraviolet light absorption of the thio ester linkage (8). The application of this assay is illustrated in Fig. 1. Under these conditions, the rate is proportional to enzyme concentration up to a change in optical density of 0.04 per minute.

Protein was determined spectrophotometrically by measuring the ab-

sorption of light at wave-lengths 280 and 260 mμ with a correction for the nucleic acid content from the data given by Warburg and Christian (9). For the early steps in the procedure, when the green color contributes markedly to the absorption, the protein concentration was determined by the biuret method (10) with crystalline ovalbumin, previously dialyzed free of salt, as a standard. The difference between the two protein determinations was usually between 10 and 15 per cent.

Preparation of Acetone Powder—Fresh spinach leaves, washed free of soil, are ground in a Waring blendor in the cold with 20 volumes of acetone. The mixture is filtered on a Büchner funnel and the green cake is treated with acetone as before. After drying at room temperature, the light green powder can be kept for months in the cold with no appreciable loss of activity.

Extraction—The acetone powder is extracted by gentle stirring with 16 volumes of 0.0167 M potassium phosphate buffer, pH 7.4, at room temperature. Excessive stirring is avoided to prevent foaming. The insoluble material is removed by centrifugation at 0° for 15 minutes at 18,000 × *g*. The clear green supernatant fluid is used immediately for the next step. All subsequent operations are carried out at 0° unless otherwise indicated.

Ammonium Sulfate Fractionation—The extract is brought to 56 per cent ammonium sulfate saturation by the slow addition of solid ammonium sulfate with mechanical stirring. The pH is kept close to 7.2 to 7.4 by the dropwise addition of 2.0 N KOH. Stirring is continued for 30 minutes and then the mixture is centrifuged at 18,000 × *g* for 30 minutes. Solid ammonium sulfate is added to the supernatant fluid to give 67 per cent saturation and the precipitate is collected by centrifugation at 18,000 × *g* for 30 minutes. The precipitate is dissolved in 0.0167 M potassium phosphate buffer, pH 7.4, and dialyzed overnight against 50 to 100 volumes of the same buffer.

First Acetone Fractionation—The protein concentration of the dialyzed solution is adjusted to about 6 to 7 mg. per ml. and the mixture is cooled to 0°. Acetone, cooled to about −30° to −40°, is added dropwise with mechanical stirring to a concentration of 44 per cent by volume. The temperature is allowed to fall to −7° to −9° and then maintained constant. The mixture is now allowed to stand overnight at −20°, and the next day it is centrifuged at 18,000 × *g* for 30 minutes. Standing overnight facilitates the centrifugation. Cold acetone is added to the supernatant fluid to 54 per cent by volume and the stirring is continued for 20 minutes. The mixture is centrifuged and the precipitate is immediately dissolved in the minimal volume of 0.0167 M potassium phosphate buffer, pH 7.0. The solution is dialyzed against the same buffer overnight and any precipitate which has formed is removed by centrifugation. The

clear solution is then frozen and kept in this state for 24 to 48 hours. Any additional precipitate which forms is removed by centrifugation and discarded.

Adsorption on Alumina Gel—The solution from the previous step, containing 3 to 4 mg. of protein per ml., is treated with alumina gel C_γ (dry weight 19.9 mg. per ml.) to 10 per cent by volume and stirred for 10 minutes. The gel is separated by centrifugation at 10,000 × *g* for 10 minutes and discarded. The volume of the clear supernatant fluid is measured and the gel is added to 20 per cent by volume, based on the measured volume at this point. After stirring for 10 minutes, the gel is collected by centrifuging for 10 minutes at 10,000 × *g*. This gel is eluted by stirring for 10 minutes with a volume of 0.1 M potassium phosphate buffer, pH 6.9, equal to the original volume of the enzyme solution. The buffer is added in small portions. The mixture is stirred thoroughly until it appears to be homogeneous before a further addition of buffer solution is made.

Second Acetone Fractionation—The eluate at pH 6.9 from the preceding step is cooled down to 0°. Acetone, cooled to −30° to −40°, is added dropwise with mechanical stirring to 50 per cent by volume. During the addition, which takes about 20 minutes, the temperature is allowed to fall to −5°, care being taken to avoid freezing. Stirring is continued for 20 minutes and then the mixture is centrifuged at −5° and 13,000 × *g* for 20 minutes. Acetone is added to the supernatant fluid to 57 per cent by volume and the precipitate is collected by centrifugation for 10 minutes at 13,000 × *g*. The precipitate is immediately dissolved in the minimal amount of 0.0167 M potassium phosphate buffer, pH 7.4, and dialyzed against the same buffer overnight. A summary of the purification is given in Table I. The highest specific activity which has been obtained by this procedure is 43. This is about 5 or 6 times higher than that of the best preparations from heart (2, 3).

The enzyme is fairly stable for several months when kept frozen. After 5 months of storage at −20° losses of about 50 per cent have been observed. The data in Table I are for a typical preparation carried through to completion within several days. If the procedure takes considerably longer, some losses in activity may be encountered on storing the enzyme between individual steps. Enzyme solutions should be stored at −20° in the frozen state. The enzyme can also be isolated from extracts of fresh spinach leaves. The specific activity of such extracts is about 0.03, which is similar to that of heart extracts (2). It should be noted that the best spinach fractions represent more than a 1000-fold purification over fresh leaf extracts. It has been observed that there is some variability in the procedure, especially in the first acetone step. It is advisable, therefore, to carry out at least this step on a small scale in a trial run before proceeding with the main batch of enzyme.

The only other enzymatic activity which has been detected in the best P enzyme fractions is ATPase. This activity can only be detected if relatively large amounts of the enzyme are used. Among the other enzymatic activities which have been looked for and found to be absent are myokinase (adenylic kinase), succinyl-acetoacetyl CoA transferase (11, 12), ITP-ADP transphosphorylase (nucleoside diphosphokinase) (13, 14), acetate-activating enzyme (15), and succinyl CoA deacylase (1, 2, 16).

Equilibrium Measurements

A direct determination of the equilibrium constant of Reaction 1 became possible with the purified spinach enzyme because of the absence of interfering myokinase. Equilibrium was established by incubating at pH 7.4

TABLE I

Purification of P Enzyme from Spinach

14,400 gm. of spinach leaves (720 gm. of acetone powder).

Step	Volume	Enzyme units	Protein	Specific activity	Yield
	ml.		*mg.*	*units per mg. protein*	*per cent*
Acetone powder extract	5000	3600	16,200	0.22	100
Ammonium sulfate fractionation (56–67)	290	2520	1,320	1.9	70
1st acetone fractionation (44–54)	95	2020	306	6.6	56
Alumina gel eluate	105	1020	75	13.5	28
2nd acetone fractionation	11	720	20	36	20

and 20° for 10 minutes. Trial runs indicated that no detectable reaction occurred after this time. The concentrations of succinyl CoA, ADP, and phosphate were determined before and after incubation as described under "Methods." The change in the concentration of these three reactants was always in good agreement. In addition, the initial concentration of CoA and ATP was determined. Since the concentration of succinate was always made large relative to the other reactants, its variation in a given experiment was neglected in the calculation of the individual equilibrium constants.

The results of a series of measurements in which the concentration of some of the reactants was varied is presented in Table II. The average value of the equilibrium constant (K = (succinate)(CoA)(ATP)/(succinyl CoA)(ADP)(P)) was 3.7. The change in free energy calculated from this value is about −750 calories. This is in agreement with results obtained with systems involving acetyl CoA which have indicated that the free energy of hydrolysis of the thio ester linkage of acyl CoA derivatives is somewhat greater than that of the pyrophosphate linkage in ATP (17, 18).

Properties of Enzyme

Enzyme-Substrate Affinity Constants—The K_m values for some of the substrates were determined under the conditions of the standard assay. The values, taken from the activity-concentration curves, are for CoA, 1×10^{-4} M; succinate, 1.5×10^{-2} M; ATP, 5×10^{-4} M; and Mg^{++}, 3.5×10^{-3} M. The value for ATP must be considered as an approximation, since there is a rather marked inhibition of the rate with concentrations of ATP higher than 5×10^{-3} M, *e.g.* 50 per cent inhibition at 9×10^{-3} M. The

TABLE II

Equilibrium Constant of Reaction 1

The reaction mixtures contained the following per ml.: 100 μmoles of Tris buffer, pH 7.4; 10 μmoles of $MgCl_2$; 0.47 mg. of P enzyme, specific activity 21; other additions as indicated. The final volume was made up with water to 1.0 ml. Incubation, 10 minutes at 20°. All concentrations are expressed as micromoles per ml.

Initial concentration						Final concentration					K
Succinyl CoA	Phosphate	ADP	Succinate	ATP	CoA	Succinyl CoA	Phosphate	ADP	ATP	CoA	
0	1.62	0.42	50	2.58	1.58	1.28	3.11	1.61	1.39	0.30	3.25
0	1.72	0.48	150	2.52	2.36	1.72	3.45	2.30	0.70	0.64	4.92
0	1.50	0.45	100	2.54	1.58	1.40	2.89	1.83	1.17	0.18	3.01
1.33	2.79	2.34	50	0.10	0	0.90	2.32	1.91	0.53	0.43	2.86
1.33	3.37	2.34	25	0.10	0	0.69	2.73	1.70	0.74	0.64	3.70
0.40	1.97	2.34	100	0.10	0	0.25	1.78	2.19	0.26	0.16	4.27
Average											3.7

affinity of the spinach enzyme for succinate appears to be somewhat lower than that of the enzyme prepared from heart (3).

Inhibitors—The inhibition of the enzyme by excess ATP has already been mentioned. By comparing the rate of the reaction under standard conditions with that observed in the absence of hydroxylamine, it is apparent that hydroxylamine inhibits the reaction. At the concentration of hydroxylamine used in the standard assay, the inhibition may be as high as 50 per cent. DNP at 1×10^{-4} M does not inhibit the spinach enzyme. It had previously been found that the heart enzyme is not inhibited by this concentration of DNP (2, 19). *p*-Chloromercuribenzoate (2×10^{-5} M) completely inhibits the reaction (7). It should be noted that the heart enzyme was not inhibited by 1×10^{-3} M iodoacetate (1).

Specificity—The enzyme appears to be quite specific for succinate. The following compounds, when incubated with the enzyme under standard conditions, did not give rise to detectable amounts of hydroxamic acid:

acetate, propionate, butyrate, lactate, malonate, fumarate, malate, tartrate, aspartate, glutarate, α-ketoglutarate, glutamate, adipate, citrate. Pantetheine cannot substitute for CoA in the reaction, even when 10 times the usual concentration of enzyme is used.

The report that the P enzyme prepared from heart reacts directly with GDP rather than with ADP (5) made it of interest to see whether the spinach enzyme showed the same nucleotide specificity. The first indication that this was not the case came from experiments with a sample of ATP purified by ion exchange chromatography (see "Methods"). It was found that a fraction eluted from Dowex 1 resin (20) with 0.15 M NaCl-0.01 N HCl showed little activity with the heart enzyme, while it was active with

TABLE III

Activity of Heart and Spinach P Enzymes with ATP Preparations of Various Degrees of Purity

The system contained the following components (in micromoles): Tris buffer, pH 7.4, 100; $MgCl_2$, 10; succinate, 100; CoA, 0.14; ATP, 0.5; sodium borohydride, 3. Heart enzyme (specific activity 1.0), 0.3 mg. Spinach enzyme (specific activity 20), 0.03 mg. Final volume 1.0 ml. Incubated for 10 minutes at 20°.

Source of enzyme	SH disappearing		
	Amorphous ATP	Rechromatographed ATP	Crystalline ATP
	μmole	*μmole*	*μmole*
Heart	0.068	0.010	0.002
Spinach	0.120	0.126	0.128

the spinach enzyme. The same was true of crystalline ATP (Table III). With this preparation, the P enzyme from heart was almost inactive. Reaction of the heart enzyme with amorphous commercial preparations of ATP is due to the contamination of these preparations with small amounts of guanosine polyphosphates and to the fact that the heart preparations used contain the nucleoside diphosphokinase catalyzing Reaction 3. As shown in Table IV, the heart enzyme is active with GDP but not with ADP (purified by ion exchange chromatography), while the reverse is true of the spinach enzyme. The heart enzyme is active with ITP, in addition to GTP (5). Fig. 1 shows that ITP cannot replace ATP for the enzyme prepared from spinach. The slight activity with ITP is due to the presence of a small amount of ATP in the ITP preparation. The failure of ITP to substitute for ATP when ADP is added also demonstrates the absence of the ITP-ADP nucleoside diphosphokinase (13, 14) from the purified spinach enzyme.

Attempts to Remove Bound Nucleotides—The best preparations of P enzyme from spinach have light absorption ratios, 280 mμ/260 mμ, close to 1.40. This is significantly lower than that obtained for the heart enzyme.[2] Proteins free of nucleic acid usually have ratios as high as 1.75 (21) and the possibility was suggested that the spinach enzyme had a bound nucleotide prosthetic group. To test this possibility, the purified enzyme was subjected to a variety of treatments which have been used to remove either nucleic acid or bound nucleotides from proteins. These included treatment with protamine, charcoal, Dowex 1 resin, Mn^{++}, dialysis against alkaline pyrophosphate buffer, and precipitation at pH 4.0 with ammonium sulfate to 40 per cent saturation. In each case, the treated protein was examined for (*a*) an increase in the 280 mμ/260 mμ ratio and (*b*) a difference in activity when assayed with crystalline and amorphous ATP. In no case was there any indication that a nucleotide had been removed. Finally, an attempt was made to assay specifically for a bound guanosine or inosine polyphosphate. 2.5 mg. of spinach enzyme, which had been carried through the whole purification procedure and which was originally of specific activity 20, were acidified to pH 2.0 with 1.0 N HCl and boiled for 10 minutes. After cooling and neutralizing, the solution was tested for GDP (or IDP) with the purified heart P enzyme (containing the nucleoside diphosphokinase) and crystalline ATP under the conditions of Table III or of the standard assay. Although this assay was sensitive to 0.003 μmole of GDP, no GDP could be detected in the treated spinach enzyme.

TABLE IV

Activity of Heart and Spinach P Enzymes with Different Nucleotides

The system contained the following components (in micromoles): Tris buffer, pH 7.4, 50; $MgCl_2$, 0.5; succinyl CoA, 0.4; ADP or GDP, 0.3; potassium phosphate, pH 7.4, 50. Spinach enzyme (specific activity 15), 0.04 mg.; heart enzyme (specific activity 1.0), 0.5 mg. Final volume 1.0 ml. Incubated for 10 minutes at 20°.

Source of enzyme	SH liberated	
	GDP	ADP
	μmole	*μmole*
Spinach	0.006	0.244
Heart	0.134	0.020

DISCUSSION

The discovery that the P enzyme from heart is specific for GTP and ITP represents the second example of a reaction which utilizes or generates

[2] Kaufman, S., unpublished experiments.

pyrophosphate energy and yet does not involve ATP directly. In the case of oxalacetic carboxylase fom chicken liver, it has been reported that ITP or IDP is the specific reactant (22). The finding that the P enzyme from spinach has a different nucleotide specificity than that from heart raises the question of the generality of this phenomenon.

The low light absorption ratio at 280 mμ/260 mμ observed with even the most active fractions of the spinach P enzyme suggested the interesting possibility that GDP (or IDP) serves as a bound prosthetic group. According to this hypothesis, what is accomplished by two separate enzymes in the heart system would be carried out by a single protein unit in the spinach. The sequence of reactions would then be

(2, *a*) Succinyl CoA + enzyme-GDP + P $\rightleftharpoons$ succinate + HS-CoA + enzyme-GTP

(3, *a*) Enzyme-GTP + ADP $\rightleftharpoons$ enzyme-GDP + ATP

The exchange of P^{32}-labeled ADP with ATP catalyzed by the spinach enzyme (6, 7) would not be inconsistent with this formulation, since Reaction 3, *a* would predict the occurrence of this exchange. There is, however, no direct experimental evidence in favor of this hypothesis. Indeed, the failure of added GDP to react in the system would be surprising if this formulation were correct. This would require that enzyme-GTP (Reaction 3, *a*) react with ADP but not with GDP. Of course, it is possible that a nucleotide other than GDP or IDP might be involved. The fact that all attempts to separate a bound nucleotide from the enzyme were unsuccessful might indicate that, if there is a nucleotide prosthetic group, it would be covalently linked to the protein.

Methods

Orthophosphate was determined by the procedure of Fiske and Subbarow (23) as modified by Lohmann and Jendrassik (24). Sulfhydryl was determined by the method of Grunert and Phillips (25) with L-cysteine as a standard. Absolute concentrations of CoA were determined as previously described (2). With CoA standardized in this way, the P enzyme was also used to determine CoA under the conditions of the standard assay. A standard curve is constructed by measuring the rate of the reaction as a function of CoA concentration. From the rate obtained with the unknown CoA solution, the CoA concentration can be interpolated from the curve. Several methods were employed to assay for ATP and ADP. The sum of ATP and ADP was determined by the use of hexokinase, glucose, glucose-6-phosphate dehydrogenase, and myokinase. ADP was determined separately with phospho(enol)pyruvate kinase and lactic dehydrogenase. The assay is based on the following reactions: phospho(enol)pyruvate + ADP $\rightleftharpoons$ ATP + pyruvate; DPNH + H^+ + pyruvate $\rightleftharpoons$ lactate + DPN^+.

The oxidation of DPNH is followed by measuring the decrease in optical density at 340 mμ in the Beckman spectrophotometer. In some of the equilibrium experiments, ADP and ATP were determined by ultraviolet absorption following chromatography on Dowex 1 resin (20). Succinyl CoA was determined with the P enzyme measuring the liberation of SH groups. The solution of succinyl CoA to be assayed is added to a mixture of 50 μmoles of potassium phosphate, pH 7.4, 2.5 μmoles of ADP, 10 μmoles of $MgCl_2$, and a large excess of P enzyme (6 to 8 units) in a volume of 1.0 ml. The mixture is incubated for 5 minutes at 20° and the amount of SH liberated is determined. This should be corrected for any SH present at zero time. GDP was assayed by using the standard P enzyme assay with crystalline ATP and purified heart enzyme (2). As already mentioned, the heart enzyme preparation used contains the ATP-GDP nucleoside diphosphokinase. The unknown solution is run with several concentrations of a standard GDP preparation and the results are plotted as a curve with rate as abscissa and GDP concentration as ordinate; the GDP concentration of the unknown solution is determined by interpolation from the curve.

Preparations—ATP, ADP, and CoA were obtained from the Pabst Laboratories. Crystalline disodium ATP was obtained from the Sigma Chemical Company. Succinyl CoA was prepared as in previous work (2) by the method of Simon and Shemin (26). A crystalline lactic dehydrogenase fraction from rabbit muscle was kindly provided by Dr. M. Schwartz. We are indebted to Dr. E. E. Snell for a sample of pantethine. Phospho(enol)pyruvate and phospho(enol)pyruvate kinase were preparations kindly supplied by Dr. A. Kornberg. A highly purified sample of GDP-mannose was provided by Dr. E. Cabib and Dr. L. F. Leloir.

Larger amounts of GDP were prepared by a modification of the method of Cabib and Leloir for GDP-mannose (27). 2 kilos of yeast were carried through all the steps of the procedure up to the chromatography on Dowex 1 resin. A column of Dowex 1 (chloride), 1.7 × 5.3 cm., was used and elutions were begun with 0.01 N HCl-0.01 M NaCl. After about 1 liter of effluent had been collected, which contained close to 80 per cent of the nucleotides, a new small peak came off which contained GDP as determined enzymatically after hydrolysis. The peak fractions were combined, brought to 90–95° for 10 minutes, and then neutralized to phenolphthalein with 2.0 N KOH in the cold. The heat treatment hydrolyzes the linkage between GDP and mannose but not the pyrophosphate linkage. 30 per cent excess barium acetate was added and then 3 volumes of cold ethanol. After neutralizing again to phenolphthalein, the mixture was stored at −20° overnight. After centrifuging, the precipitate was washed with alcohol and ether and dried.

Commercial amorphous ATP can be made essentially free of inosine and guanosine nucleotides (as assayed with heart P enzyme) by chromatography on Dowex 1 (chloride) (20). After the ADP is eluted with 0.01 N HCl-0.02 M NaCl, the column is perfused with 0.01 N HCl-0.15 M NaCl. The first fractions eluted with this mixture were found to be almost without activity for the heart enzyme, while still active with the spinach enzyme (see Table III).

SUMMARY

The purification from spinach of the P enzyme, catalyzing the reversible conversion of succinyl coenzyme A, adenosine diphosphate, and orthophosphate to succinate, coenzyme A, and adenosine triphosphate, is described; the equilibrium constant of the reaction has been determined. Some of the properties of the enzyme are described. The most striking difference between the spinach and the heart P enzymes is in nucleotide specificity. While the heart enzyme is specific for guanosine or inosine di- or triphosphate, the spinach enzyme reacts only with adenosine di- or triphosphate.

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STUDIES ON THE MECHANISM OF THE REACTION CATALYZED BY THE PHOSPHORYLATING ENZYME*

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The purification and properties of the phosphorylating enzyme (P enzyme) from heart muscle (2) and from spinach leaves (3) have been described. The spinach enzyme has been shown to catalyze the reversible Reaction 1. In the heart system the nucleoside diphosphate reactant is GDP (or IDP) rather than ADP (4).[1] ATP is formed through a secondary reversible transphosphorylation between GTP (or ITP) and ADP (Reaction 2) catalyzed by a separate enzyme (4) analogous to the nucleoside diphosphokinase (5, 6) of Krebs and Hems and Berg and Joklik.

(1) $$\text{Succinyl-S-CoA} + \text{ADP} + \text{P} \xrightleftharpoons{(Mg^{++})} \text{succinate} + \text{HS-CoA} + \text{ATP}$$

(2) $$\text{GTP (or ITP)} + \text{ADP} \rightleftharpoons \text{GDP (or IDP)} + \text{ATP}$$

Although the P enzyme has been extensively purified from two different sources, and the over-all reaction is well understood, little is known about the reaction mechanism. This has now been studied with the purified spinach enzyme. It has been found that the enzyme catalyzes a rapid exchange of succinate with succinyl CoA and of ADP with ATP. These results, and others to be discussed, suggest that a phosphorylated CoA derivative may participate in the reaction.

Incorporation of P^{32}-Labeled Orthophosphate in ATP—It has previously

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[1] The following abbreviations are used: CoA or CoA-SH, coenzyme A (reduced); succinyl CoA or succinyl-S-CoA, succinyl coenzyme A; succinyl P, succinyl monophosphate; acetyl CoA, acetyl coenzyme A; ATP, GTP, and ITP, adenosine, guanosine, and inosine triphosphate; ADP, GDP, and IDP, adenosine, guanosine, and inosine diphosphate; AMP, adenosine-5′-phosphate; GSH, reduced glutathione; Tris, tris(hydroxymethyl)aminomethane; P, orthophosphate.

been found that the heart P enzyme does not catalyze the incorporation of orthophosphate in ATP unless both CoA and succinate are present (2). As shown in Table I, this is also true of the spinach enzyme. Under these conditions, the incorporation of phosphate in ATP merely reflects the over-all reversibility of the reaction.

Exchange of C^{14}-Succinate with Succinyl CoA—The spinach enzyme has been found to catalyze a rapid exchange of C^{14}-succinate with succinyl CoA under conditions in which no net chemical change occurs. As presented in Table II, this exchange is stimulated by orthophosphate and requires the presence of Mg^{++}.[2] The addition of ADP, which completes the over-all reaction system (*cf.* Reaction 1), leads to a small but significant inhibition

TABLE I

Incorporation of Radioactive Phosphate in ATP

The complete system contained the following components (in micromoles): Tris buffer, pH 7.4, 100; $KH_2P^{32}O_4$, 5; $MgCl_2$, 10; GSH, 10; ATP, 5; succinate, 100; CoA, 0.7. Spinach P enzyme, specific activity 10, 0.67 mg. Final volume 1.0 ml. Incubated for 15 minutes at 25°.

System	Final specific radioactivity		Exchange
	P	ATP	
	c.p.m. per μmole	*c.p.m. per μmole*	*per cent*
Complete	12,500	3250	31
No succinate	16,400	0	0
" CoA	16,500	0	0
" succinate, no CoA	16,500	0	0

of the exchange. The purified spinach enzyme is completely free of CoA transferase (7, 8) which catalyzes a similar exchange in the absence of orthophosphate and Mg^{++} (*cf.* (9)). It should be noted that, even in the absence of added phosphate, the system contains small amounts present as a trace contaminant in the succinyl CoA. This probably accounts for the failure to find a more complete dependence of the rate of exchange on added phosphate.

Exchange of P^{32}-Labeled ADP with ATP—The purified spinach enzyme catalyzes a rapid exchange of ADP^{32} with ATP in the absence of added components other than the two nucleotides and Mg^{++}. Fig. 1 shows the effect of enzyme concentration on the rate of exchange and the complete Mg^{++} dependence of the reaction. The same rates of exchange are obtained

[2] Similar results have previously been obtained in this laboratory with P enzyme from pig heart. These experiments were originally planned and carried out by Dr. Charles Gilvarg.

with ADP and ATP made essentially free of other nucleotide contaminants by ion exchange chromatography (*cf.* (3)). The purified enzyme is free of myokinase (adenylic kinase) and of nucleoside diphosphokinase (4–6), either of which could lead to labeling of ATP. The absence of diphospho-

TABLE II

Exchange of C^{14}-Labeled Succinate with Succinyl CoA

The complete system contained the following components (in micromoles): Tris buffer, pH 7.4, 50; $MgCl_2$, 5; orthophosphate, 5; succinyl CoA, 1.3; 2-C^{14}-succinate, 5. Spinach enzyme, specific activity 30, 0.17 mg.; ADP, 2.5 mg., added as indicated. Final volume 1.0 ml. Incubated for 6 minutes at 20°.

System	Final specific radioactivity		Exchange
	Succinic acid	Succinohydroxamic acid	
	c.p.m. per μmole	*c.p.m. per μmole*	*per cent*
Complete	11,800	1663	14.4
" + ADP	11,800	1208	10.4
No phosphate	12,000	629	5.4
" Mg^{++}	12,200	45	0.4

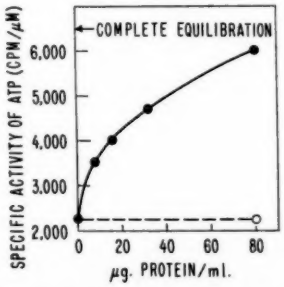

FIG. 1. Rate of ADP-ATP exchange as a function of P enzyme concentration. The complete system (●) contained (in micromoles) Tris buffer, pH 7.4, 50; $MgCl_2$, 50; ADP^{32} (adenosine-P-P^{32}), 1.0; ATP, 1.0; and spinach P enzyme, specific activity 14, as indicated. Final volume, 1.0 ml. Incubated for 10 minutes at 20°. ○, system without $MgCl_2$. The spinach enzyme was dialyzed free of phosphate before use. The specific radioactivity of ADP at the end of incubation varied between 6000 and 8400 c.p.m. per μmole in the individual experiments.

kinase was shown by direct enzymatic assay (3). The freedom from myokinase was demonstrated by (*a*) lack of AMP formation during the experiments, (*b*) failure to obtain labeled ATP in experiments in which ATP was omitted from the reaction mixture and added only as a carrier prior to chromatography, (*c*) failure of ADP to replace ATP for the P enzyme

reaction in the standard assay (3), and (*d*) negative reaction for myokinase on assay of the spinach enzyme with ADP in the presence of hexokinase, glucose, glucose-6-phosphate dehydrogenase, and triphosphopyridine nucleotide.

The only known enzymatic contaminant in the purified P enzyme is an ATPase. Although present only in trace amounts, it was conceivable that the exchange of ADP^{32} with ATP might be catalyzed by this enzyme rather than by the P enzyme itself. To test this possibility the occurrence of the

TABLE III

Exchange of ADP^{32} with ATP Studied with Myosin ATPase and Crystalline Hexokinase

The complete system in the experiment with ATPase contained the following components (in micromoles): Tris buffer, pH 9.0, 100; KCl, 150; $CaCl_2$, 10; ADP^{32}, 1.0; ATP, 5.0. Enzyme, 0.06 mg. Final volume 1.0 ml. Incubated for 30 minutes at 37°. In the hexokinase experiment (in micromoles): Tris buffer, pH 7.4, 50; $MgCl_2$, 5.0; ADP^{32}, 1.2; ATP, 0.9; glucose, 20. Crystalline hexokinase (dialyzed against 0.0167 M potassium phosphate buffer, pH 6.9, to remove glucose) 0.2 mg. Final volume 1.0 ml. Incubated for 5 minutes at 20°.

Enzyme	System	Final specific radioactivity	
		ADP	ATP
		c.p.m. per μmole	*c.p.m. per μmole*
ATPase	No enzyme	55,600	760
	" ATP*	55,600	580
	Complete	29,200	2,060
Hexokinase	No enzyme	32,800	780
	" glucose	35,900	1,330
	Complete	21,000	10,700†

* ATP added prior to chromatography.

† Approximate value because the amount of ATP remaining was too small to be accurately determined.

ADP-ATP exchange was studied with myosin ATPase. The results, presented in Table III, demonstrate that the relatively small exchange observed with ATPase could not be responsible for that observed with the purified spinach enzyme (*cf.* Fig. 1). Moreover, it is questionable whether the exchange obtained with the myosin preparation is actually due to ATPase. A similar lack of exchange of ADP^{32} with ATP has recently been observed by Koshland *et al.* and by Gergely and Jencks (see (10)).

The possibility was considered that the ADP-ATP exchange might be catalyzed by phosphate-transferring enzymes in general. However, negative results have been obtained in this laboratory (11) with acetokinase, the enzyme catalyzing the reversible transfer of phosphate from ATP to

acetate. Investigation of the occurrence of the exchange with crystalline hexokinase showed that there was a very small exchange in the presence of Mg^{++}, ADP^{32}, ATP, and hexokinase, although marked exchange occurred when the system was completed by the addition of glucose (Table III). The conversion of labeled ADP to ATP in the latter case demonstrates that the reaction catalyzed by hexokinase is reversible (Reaction 3).[3]

(3) $$\text{ATP} + \text{glucose} \rightleftharpoons \text{ADP} + \text{glucose-6-phosphate}$$

The fact that the catalysis of the ADP-ATP exchange by P enzyme preparations appears to be proportional to their specific activity makes it likely that the exchange is related to the intrinsic activity of the enzyme.

TABLE IV

Exchange of ADP^{32} with ATP with Various P Enzyme Preparations

The system contained the following components (in micromoles): Tris buffer, pH 7.4, 50; $MgCl_2$, 5.0; ADP^{32}, 1.0; ATP, 1.0; enzymes as shown. Final volume 1.0 ml. Incubated for 10 minutes at 20°. The enzymes had been kept frozen for about 3 months, with occasional thawing, and had lost activity to varying degrees. The initial specific activity of the spinach enzyme Preparation A was 27; that of Preparation B, 44.

Enzyme			Specific radioactivity of ATP	
Preparation	Specific activity	Amount used		
	units per mg. protein	*units*	*c.p.m. per μmole*	*c.p.m. per μmole per unit of enzyme*
A	7.0	0.154	8770	57,000
B	28.0	0.137	7310	53,500

Comparison of two different preparations of spinach P enzyme showed that they catalyzed the exchange at the same rate per unit of P enzyme (Table IV). Further indication that the exchange is related to the P enzyme activity might be seen in the fact that it is inhibited by CoA (Table V). The effect of CoA is quite specific since, as demonstrated in Table V, acetyl CoA, oxidized CoA, reduced glutathione, and pantetheine are without effect.

With crude preparations of the spinach enzyme, *e.g.* the first acetone fraction (3), the rate of the ADP-ATP exchange per unit of P enzyme is much faster than with the more highly purified preparations. The faster rate of exchange with these fractions may be contributed by nucleoside diphosphokinase or other enzymes. Enzymes catalyzing the reversible transfer of the terminal phosphate of certain nucleoside diphosphates to

[3] The same conclusion has been reached independently by Gamble and Najjar (12) on the basis of experiments with C^{14}-labeled glucose.

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TABLE V

Effect of Various Compounds on Exchange of ADP^{32} with ATP Catalyzed by Spinach P Enzyme

The complete system contained the following components (in micromoles): Tris buffer, pH 7.4, 50; $MgCl_2$, 5.0; ADP^{32}, 1.5; ATP, 4.5. Enzyme, specific activity 30, 0.012 mg. 10 μmoles of succinate, 5 μmoles of GSH, and 0.7 μmole of CoA-SH, CoA derivatives, or pantethine added when indicated. Final volume 1.0 ml. Incubated for 10 minutes at 20°. The specific radioactivity of the ADP after incubation varied between 11,000 and 12,000 c.p.m. per μmole.

System	Specific radioactivity of ATP	Exchange
	c.p.m. per μmole	*per cent*
Complete	760	22.8
" + succinate	790	23.8
" + CoA	360	10.9
" + acetyl CoA	770	23.3
" + oxidized CoA	710	21.4
" + GSH	750	22.6
" + " + pantethine	780	23.4
No enzyme	84	

TABLE VI

Effect of p-Chloromercuribenzoate

The over-all reaction (Reaction 1) was measured by the formation of SH (free CoA) in the presence of succinyl CoA (0.4 μmole), ADP (3.0 μmoles), potassium phosphate, pH 7.4 (50 μmoles), $MgCl_2$ (5.0 μmoles), Tris buffer, pH 7.4 (50 μmoles), and enzyme, specific activity 23 (0.01 mg.). Final volume 1.0 ml. Incubated for 10 minutes at 20°. SH was determined as in the preceding paper (3). For succinyl CoA arsenolysis the conditions were the same, except for the presence of potassium arsenate (50 μmoles) and the omission of ADP and orthophosphate. The system for the exchange experiment consisted of Tris buffer, pH 7.4 (100 μmoles), $MgCl_2$ (5.0 μmoles), ADP^{32} (0.3 μmole), ATP (0.6 μmole), and enzyme, specific activity 28 (0.036 mg.). Final volume 1.0 ml. Incubated for 20 minutes at 20°. Spinach P enzyme used in all cases.

Reaction	p-Chloromercuribenzoate concentration	Inhibition
	moles per l.	*per cent*
Over-all	1×10^{-5}	13
"	1×10^{-5}	29
"	2×10^{-5}	60
"	5×10^{-5}	100
Arsenolysis	5×10^{-5}	100
ADP^{32}-ATP exchange	5×10^{-5}	0

ADP (13) would bring about the ADP-ATP exchange in the presence of catalytic amounts of nucleoside diphosphate. The ADP-ATP exchange has also been observed with the enzyme catalyzing the synthesis of glutathione from ATP, γ-glutamylcysteine, and glycine, when the enzyme is incubated with ADP^{32}, ATP, and Mg^{++} (14). The presence in the purified spinach preparations of contaminating enzymes which may cause an ADP-ATP exchange has only been excluded for myokinase and nucleoside diphosphokinase. However, it would appear from the observations reported in the preceding paragraph that the P enzyme itself is involved in the exchange observed with these preparations.

Effect of p-Chloromercuribenzoate—The over-all reaction catalyzed by the P enzyme is completely inhibited by 5×10^{-5} M *p*-chloromercuribenzoate (Table VI). It was of interest to determine the sensitivity of the arsenolysis of succinyl CoA, which the P enzyme has been shown to catalyze (15), to this reagent. As shown in Table VI, the arsenolysis is also completely inhibited by 5×10^{-5} M *p*-chloromercuribenzoate. On the other hand, the ADP-ATP exchange is not affected.

DISCUSSION

The simplest assumption that could be made regarding the mechanism of the reaction catalyzed by the spinach P enzyme is represented by the Reaction sequences 4 and 5. However, both of these sequences can be ruled out.

(4, *a*) Succinyl CoA + P ⇌ succinyl P + CoA

(4, *b*) Succinyl P + ADP ⇌ succinate + ATP

(5, *a*) Succinyl CoA + P ⇌ succinate + CoA-P

(5, *b*) CoA-P + ADP ⇌ CoA + ATP

Reaction 4 can be excluded for the following reasons: Reaction 4, *a* would be analogous to that catalyzed by phosphotransacetylase (16) and would be expected to lead to an arsenolysis of succinyl P in the presence of the enzyme and CoA. This is not the case, as shown in experiments with synthetic succinyl P. In addition, Reaction 4, *b* has been tested directly by looking for a transfer of P from succinyl P to ADP. This reaction is not catalyzed by the spinach P enzyme. The results of the ADP-ATP exchange would also not be in agreement with Reaction 4, as no succinate is required for this exchange. Furthermore, if Reactions 4, *a* and 4, *b* occurred (the latter being analogous to the reaction catalyzed by acetokinase (11)), the incorporation of C^{14}-succinate in succinyl CoA should require both phosphate and ADP, but, as already pointed out, the latter is not required. Reaction sequence 5 can also be ruled out for, in this case,

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the ADP-ATP exchange would require CoA; this is not the case. Indeed, CoA actually inhibits the exchange (Table V).

A reaction sequence which is consistent with all the experimental results is Reaction 6 where E(CoA-P) represents a phosphorylated derivative of CoA, which might be bound to the enzyme, and E[P] stands for an enzyme-phosphate compound.

(6, *a*) $\text{Succinyl CoA} + \text{P} + \text{E} \rightleftharpoons \text{succinate} + \text{E(CoA-P)}$

(6, *b*) $\text{E(CoA-P)} \rightleftharpoons \text{E[P]} + \text{CoA}$

(6, *c*) $\text{E[P]} + \text{ADP} \rightleftharpoons \text{ATP} + \text{E}$

It can be seen that, according to this scheme, the incorporation of orthophosphate into ATP would require CoA and succinate, the incorporation of succinate into succinyl CoA would require phosphate, and the exchange of ADP with ATP would take place with no other additions but the enzyme. The specific inhibition by CoA of the latter reaction could be explained by competition between CoA and ADP for E[P] and would provide indirect evidence for the occurrence of Reaction 6, *b*. In an analogous manner, the inhibition of the succinate exchange by ADP could be explained by assuming that the steady state concentration of E(CoA-P) is decreased in the presence of ADP owing to the occurrence of Reactions 6, *b* and 6, *c*.

Although convincing evidence has been obtained against the participation of succinyl P in the reaction as a free intermediate, its occurrence as an enzyme-bound compound has not been ruled out. If, however, such an enzyme-succinyl P complex were even slightly dissociable, C^{14}-succinate should be incorporated in succinyl P added to the reaction mixture. In experiments with synthetic succinyl P and labeled succinate, no such incorporation was observed. This result, together with the fact that, contrary to CoA, succinate has no effect on the ADP-ATP exchange (Table V), makes the scheme involving E(CoA-P) more likely. The formation of an S-phosphoryl CoA has been postulated by Lynen *et al.* (17) for a similar reaction, *i.e.* that catalyzed by the acetate-activating enzyme, but later work suggested a different mechanism in this case (18).

It may be added that a sequence involving reaction of succinyl CoA with ADP rather than with orthophosphate can be excluded because, were this the case, the incorporation of C^{14}-succinate in succinyl CoA would require either ADP or both orthophosphate and ADP. It should be emphasized that the arsenolysis of succinyl CoA (15) provides further support for the view that, starting with succinyl CoA, the initial step involves a primary reaction between orthophosphate and succinyl CoA, since arsenolysis probably occurs through a replacement of phosphate by arsenate. In terms of

Reaction 6, *a* the arsenolysis of succinyl CoA might be visualized as a cleavage of the thio ester by arsenate to form succinate and arsenoyl CoA, followed by spontaneous hydrolysis of the latter.

The ADP-ATP exchange has been interpreted as indicating that the enzyme is phosphorylated. No information is available which would suggest the nature of the group on the protein which is involved in this reaction. The finding that the exchange is unaffected by *p*-chloromercuribenzoate would make —SH participation unlikely. The possibility has already been considered (3) that a nucleotide prosthetic group may be phosphorylated in this reaction. In contrast to Reaction 6, *c*, Reaction 6, *a* would seem to require —SH groups, since the arsenolysis of succinyl CoA is sensitive to *p*-chloromercuribenzoate.

It has so far not been possible to resolve the spinach P enzyme into separate components. Attempts to demonstrate the accumulation of a free reaction intermediate have been unsuccessful (*cf.* also (19)). The failure of an intermediate to accumulate might be due either to a very unfavorable equilibrium of the partial reactions in either direction or to the intermediate occurring in a non-dissociable protein-bound form.

Methods

In the experiments on the exchange of C^{14}-succinate with succinyl CoA, the thio ester was converted to succinohydroxamic acid by addition of hydroxylamine and the succinic and succinohydroxamic acids were separated by ion exchange chromatography on a column (1.2 × 2 cm.) of Dowex 1 resin (chloride form). This method was developed by Dr. Charles Gilvarg. The hydroxylamine solution was made relatively salt-free as follows: 1.5 ml. of 4.0 M NH_2OH-HCl were mixed with 1.0 ml. of 7.0 N KOH and 1.0 ml. of the neutral solution was treated with 4.5 ml. of ice-cold ethanol. The precipitated salts were removed by centrifugation and the supernatant fluid was concentrated to 1.0 ml. under reduced pressure. An amount of the hydroxylamine solution containing about 600 μmoles of hydroxylamine was added to each reaction mixture after incubation and, after standing for 15 minutes at room temperature, ammonium hydroxide was added to make its concentration approximately 1.0 N and the solution was transferred to the column. The column was washed with water and then eluted with 0.01 N acetic acid; fractions of about 5.0 ml. were collected. All of the succinohydroxamic acid is usually eluted with 25 to 35 ml. of solvent. The succinate is then eluted with 0.1 N acetic acid made 0.05 M with respect to potassium chloride. The concentration of succinohydroxamic acid is determined colorimetrically with the ferric chloride reagent of Lipmann and Tuttle (20). In the experiments on incorporation of P^{32}-labeled orthophosphate in ATP and on ADP^{32}-ATP exchange, ortho-

phosphate, ATP, and ADP were separated through ion exchange chromatography by the Cohn and Carter procedure (21). Radioactivity determinations were made with a windowless flow counter.

Preparations—ADP^{32} labeled with P^{32} in the terminal phosphate group was prepared from ATP^{32} in which the two terminal phosphates were labeled. The labeled ATP was prepared through the phosphorylation of ADP, in the presence of P^{32}-labeled orthophosphate, coupled with the oxidation of α-ketoglutarate by rat liver mitochondria. The mitochondria were prepared by the method of Schneider (22). Because of the presence of myokinase in the mitochondria, the procedure yields a mixture of ATP^{32} (adenosine-P-P^{32}-P^{32}), ADP^{32} (adenosine-P-P^{32}), and non-labeled AMP. The ice-cold components of the system were measured into a conventional Warburg flask placed in ice. The reaction mixture contained the following components (in micromoles): Tris buffer, pH 7.4, 100; $KH_2P^{32}O_4$, 50; $MgSO_4$, 20; KCl, 75; AMP, 25; ADP, 6.4; cytochrome *c*, 0.05; potassium α-ketoglutarate, 100; mitochondrial suspension (equivalent to about 20 mg. of protein); and water to a final volume of 3.0 ml. A KOH-soaked filter paper was placed in the center well and the reaction was started by tipping in the α-ketoglutarate from the side bulb. Incubation was carried out at 15° and the reaction was followed by measuring the oxygen uptake. After about 90 minutes, the reaction mixture was transferred to a centrifuge tube, brought to 100° for 90 seconds, and centrifuged. The sediment was washed twice on the centrifuge each time with 3.0 ml. of water and the washings were combined with the original supernatant fluid. The solution was made up to 18.0 ml. with water and treated with ammonium hydroxide to give 1.0 N final concentration. The mixture was then chromatographed on a Dowex 1 (chloride) column according to Cohn and Carter (21). The ADP fraction was slightly contaminated with radioactive orthophosphate. The ATP fraction, which was free of orthophosphate, was concentrated by evaporation under reduced pressure and reacted with glucose, in the presence of crystalline hexokinase and Mg^{++}, to give a mixture of ADP^{32} and P^{32}-labeled glucose-6-phosphate, which were separated by chromatography on Dowex 1 (chloride). Glucose-6-phosphate is eluted together with AMP in the Cohn and Carter procedure (0.003 N HCl as eluting solvent).

Oxidized CoA was prepared by treating 0.5 ml. of a neutral solution of CoA, containing 10 mg. per ml., with 0.04 ml. of 6 per cent H_2O_2. After 15 minutes at room temperature there were no detectable —SH groups, as determined by the method of Grunert and Phillips (23). The residual H_2O_2 was destroyed by incubation with an excess of crystalline catalase and the mixture was brought to 100° for 1 minute. Acetyl CoA and succinyl CoA were prepared as previously described (2) according to Simon and Shemin (24). ATP, essentially free of guanosine and inosine nucleotides,

was prepared by ion exchange chromatography of commercial ATP as described in the preceding paper (3). Succinyl phosphate was prepared synthetically by reaction of succinic anhydride with potassium phosphate (25). Crystalline ox liver catalase was a preparation of Dr. S. Korkes. I am indebted to Dr. M. Kunitz for a generous gift of twice recrystallized yeast hexokinase, to Dr. J. Gergely for a sample of purified myosin, to Dr. E. E. Snell for a sample of pantethine, and to Dr. D. Shemin for a gift of methylene-labeled C^{14}-succinate. Other preparations, including CoA, ADP, ATP, and cytochrome *c*, were obtained commercially.

SUMMARY

The purified spinach P enzyme, which catalyzes the Mg^{++}-dependent over-all reaction succinyl coenzyme **A** (succinyl-S-CoA) + adenosine diphosphate (ADP) + orthophosphate (P) $\rightleftharpoons$ succinate + coenzyme A (HS-CoA) + adenosine triphosphate (ADP), brings about (*a*) an incorporation of P in ATP, (*b*) an exchange of radioactive succinate with succinyl-S-CoA, and (*c*) an exchange of radioactive ADP with ATP. While (*a*) requires the presence of all components of the system, reflecting the known reversibility of the over-all reaction, (*b*) only requires enzyme, Mg^{++}, orthophosphate, succinate, and succinyl-S-CoA, and (*c*) requires enzyme, Mg^{++}, ADP, and ATP only.

The mechanism of the P enzyme reaction is discussed in the light of the above results. A phosphorylated coenzyme A derivative, possibly in a non-dissociable protein-bound form, is suggested as an intermediate.

Incidentally to the P enzyme work it has been shown that crystalline hexokinase catalyzes the incorporation of P^{32}-labeled ADP in ATP when glucose is present. This demonstrates the reversibility of the reaction catalyzed by hexokinase.

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POLAROGRAPHIC DETERMINATION OF OXYGEN TENSION OF WHOLE BLOOD*

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The purpose of this study is to establish a procedure for the *direct* determination of oxygen tension of whole blood that requires a smaller sample and simpler manipulation than methods previously reported (1). A procedure has been worked out for determining dissolved plasma oxygen which proved satisfactory in our investigations. Since the success of the application of the proposed polarographic methods depends upon the proper construction of the apparatus and upon the technique, these aspects of our work are described in detail. Statistical analysis of the precision of the method is also reported.

Apparatus

The complete apparatus, consisting of five units, is shown schematically in Fig. 1. The designation and function of these units is as follows: (*A*) *polarograph* for the automatic recording of the diffusion current due to dissolved oxygen; (*B*) *electrodes and sample cell* for the application of electric potential to the dropping mercury electrode in the plasma without contact with room air; (*C*) *tonometer box* for the transfer of the blood sample from syringe to sample cell and the equilibration of the sample with the standard gas (gas of known oxygen concentration) at the constant temperature of 37°; (*D*) *water bath* for heating the standard gas while bubbling through gas washers; and (*E*) *centrifuge box* for the maintenance of constant temperature while the sample cell is being centrifuged to separate blood cells from plasma.

(*A*) *Polarograph*—A Leeds and Northrup *electrochemograph type E* is used to record the diffusion current from -0.4 to -0.6 volt with respect to the calomel half cell. The use of this automatic recording device not only releases the operator from the difficult task of taking current readings

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which change rapidly, but also gives instant visual indication on the chart as to whether the electrical system is working properly.

(*B*) *Electrodes and Sample Cell*—One of the leads from the polarograph is connected to a platinum electrode on the top of the mercury leveling

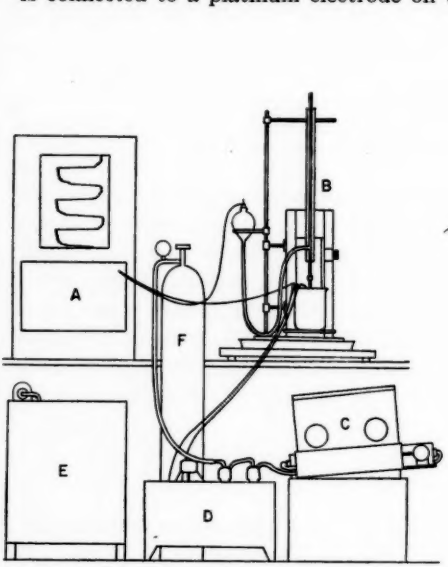

FIG. 1

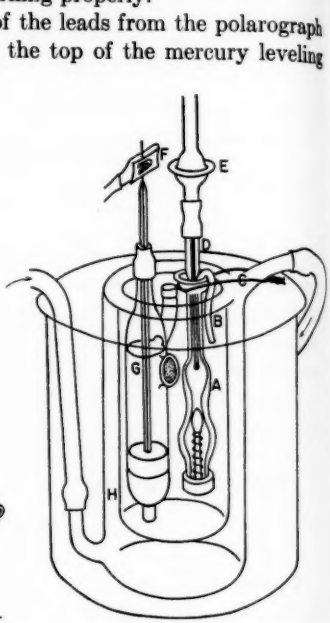

FIG. 2

FIG. 1. Schematic diagram of the apparatus for polarographic determination of dissolved oxygen in blood plasma. *A*, recording polarograph; *B*, dropping mercury electrode and accessories; *C*, tonometer box; *D*, water bath; *E*, centrifuge box; *F*, tank of analyzed gas mixture.

FIG. 2. Electrodes and sample cell. *A*, sample cell in salt bath (blood cells and plasma not shown); *B*, curved agar bridge; *C*, platinum wire loop; *D*, capillary of the dropping mercury electrode; *E*, ball and socket joint (clamp not shown); *F*, connecting clamp of the lead from the polarograph; *G*, calomel half cell; *H*, water jacket of the saturated potassium chloride bath.

bulb. The electrode, being in contact with the mercury in the bulb, is electrically connected to the mercury drops that form at the bottom of the capillary dropping electrode located directly below the mercury column. The other wire from the polarograph is attached to the calomel half cell, as shown in Fig. 2, *F*.

The dropping mercury electrode (Fig. 2, *D*) is made from capillary tubing (5 mm. outside diameter, 0.2 mm. bore) drawn out after heating in a hot flame to approximately 1 mm. outside diameter. The capillary section

is cut to about 4 to 5 cm. so that the tip of the capillary reaches the center of the upper compartment of the sample cell (Fig. 2, *A*), while the shoulder nearly closes off the opening of the neck of the sample cell. The bore of the capillary is coated with silicone (Desicote, Beckman Instruments, Inc.) and dried after rinsing with distilled water.

The calomel half cell (Fig. 2, *G*), to be used immersed, has a side arm with a rubber cap for filling the cell with the chemical constituents and a fritted glass window of fine porosity. Behind the window (9 mm. diameter) is an agar plug. When the sample cell and the calomel half cell are both immersed in the saturated potassium chloride bath, electrical contact between the plasma in the sample cell and the platinum lead of the calomel half cell is achieved by a curved agar bridge (Fig. 2, *B*) which hangs onto the lip of the socket part on top of the sample cell and dips into the salt bath. The upper end (not shown) of the glass enclosure of the agar bridge is shaped to curve half way around the capillary electrode so that a small amount of overflow plasma (when the electrode is inserted into the sample cell) is sufficient for good electrical contact. The bridge is kept in a moist vessel when not in use.

The saturated potassium chloride solution (salt bath) is maintained at 37° by circulating water through the water jacket (Fig. 2, *H*) pumped from a 38° water bath (Fig. 1, *D*). The salt bath is supported inside a large beaker which can be raised or lowered by a rack and pinion-controlled platform (Fig. 1, *B*). The stand, which supports the mercury column and the leveling bulb as well as the adjustable platform, in turn rests on a vibration-reduced stand. The vibration due to the circulating pump as transmitted through the connecting Tygon tubes does not seem to affect the polarogram, and therefore the water circulation may be maintained throughout the procedure.

The *sample cell* (Fig. 2, *A*) is constructed in such a manner that the plasma from a blood sample, once the blood is introduced into the cell, may be separated from the cells anaerobically and made ready for polarography without the need for transfer into another vessel. The glass part of the sample cell is made from a ball and socket joint (standard taper 12/2). The capillary of the socket joint is thickened and two compartments blown to the contour shown in Fig. 2. The total length is approximately 9 cm. The pear-shaped upper compartment has no projections to detain any blood cells during centrifugation. The volume ratio of the upper compartment to the lower compartment with the glass valve is approximately 1:2, which allows collection in the upper compartment of plasma free from cells. The glass valve, which is ground to fit the constricted part of the sample cell, is maintained in the "closed" position (as shown) by a steel spring. During centrifugation of the sample cell, however, the valve is opened by cen-

trifugal force to allow the sedimentation of cells into the lower part and later returns to the "closed" position when the centrifuge slows down to stop. The steel spring is held against the glass valve by a rubber cap made from a serum bottle cap. This cap may be removed easily for cleaning the sample cell. The stem of a ball joint (standard taper 12/2) is completely removed, and the cut is ground flat to insure a good fit between the bore of the ball joint and the needle hub during the transfer of the blood sample. The ball joint (not shown) is lightly greased to fit the socket so that the sample cell may be sealed after the introduction of the blood sample by simple rotation of the ball joint; it is removed from the socket just before the dropping mercury electrode is inserted into the sample cell.

(*C*) *Tonometer Box*—As shown in Fig. 1, *C*, this unit consists of a deep wooden cover with armholes on the front panel and a wooden tray beneath

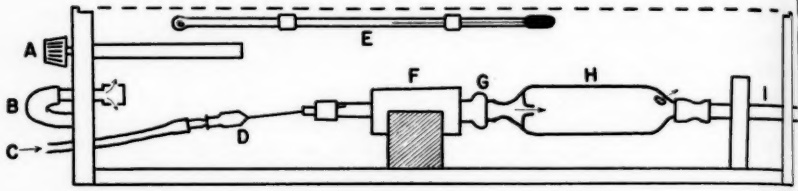

FIG. 3. Lower half of the tonometer box. *A*, thermoswitch; *B*, warm air inlet; *C*, inlet for standard gas; *D*, hypodermic needle; *E*, thermometer; *F*, thick walled rubber tube; *G*, standard tapered joint; *H*, glass tonometer; *I*, drive shaft of the motor.

(Fig. 3). The top of the wooden cover is made of double sheets of Plexiglas with 2 cm. of air space between the sheets as insulation. The armholes are just large enough to admit hands into the constant temperature box for such manipulations as transfer of blood sample from the sampling syringe into the sample cell. It might be pointed out here that control of the temperature at 37° is essential throughout the procedure to prevent the redistribution of oxygen between plasma and erythrocytes. The air stream which maintains the temperature of the box is heated by passage over a heating tape wound on a glass core 1.5 × 70 cm., enclosed in an outer glass tube of 5 cm. diameter (not illustrated). There are rubber stoppers at both ends, through one of which is an air inlet and through the other an outlet. The heating tape used is of the uninsulated type, 2.5 × 90 cm., and has a power rating of 50 watts at 110 volts. Compressed air flows through the system at the rate of 100 liters per minute. A similar air-heating unit for the centrifuge is illustrated in Fig. 4, *E*. Where the heated air enters the tray (Fig. 3, *B*), there is a thermoswitch (Fenwal,

Ashland, Massachusetts) nearby (Fig. 3, *A*) which is adjusted to maintain the temperature inside the tonometer box at 37° ± 0.1.

The standard gas, saturated with water vapor and to be used for calibration, passes from the gas washer in the water bath (Fig. 1, *D*) to the tonometer box through insulated Tygon tubing (Fig. 3, *C*). Through a hypodermic needle (Fig. 3, *D*) the gas flows on past a standard tapered joint 7/12 (Fig. 3, *G*) held by a thick walled rubber tube (Fig. 3, *F*) which is clamped on a wooden support. The inner joint is the gas inlet of the glass tonometer, as illustrated in Fig. 3. The interior of the tonometer has been coated with silicone to reduce the possibility of hemolysis when

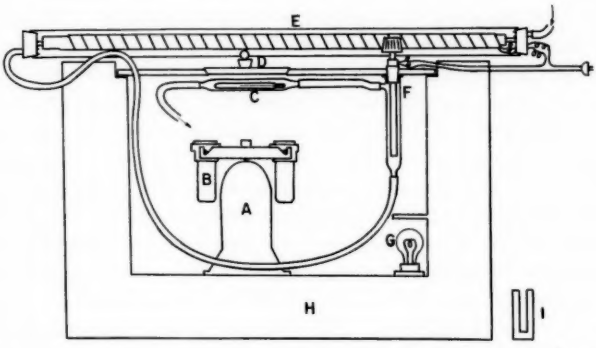

FIG. 4. Centrifuge box. *A*, centrifuge; *B*, shield for Lucite cylinder (*I*); *C*, thermometer in glass tube; *D*, transparent cover for armhole in the Transite roof; *E*, air-heating unit; *F*, thermoswitch in the air stream; *G*, tungsten light bulb; *H*, insulated box; *I*, Lucite cylinder for holding sample cell.

blood is rotated in it. Rotation of the tonometer is provided by connecting the other end of the tonometer to the drive shaft (Fig. 3, *I*) by a short piece of Tygon tubing. An electric motor turns the drive shaft at approximately 60 r.p.m. The tonometer box is supported at an angle of 7° to the horizontal (Fig. 1, *C*) so that the blood sample cannot be blown out of the tonometer by the equilibrating gas.

(*D*) *Water Bath*—The water bath is maintained at 38° to provide water for the water jacket of the saturated potassium chloride bath and to heat the standard gas bubbling through the gas washers (Fig. 1, *D*). Two 125 ml. gas washers, each filled two-thirds full with distilled water, are connected in series. By passing through the gas washers, the standard gas becomes saturated with water vapor and will not alter the water content of the blood during the equilibration. Physiological saline, which seemed to be the logical phase for the equilibration, has been found unsatisfactory

owing to the apparent drying effect of the equilibrated standard gas on the sample in the tonometer.

(*E*) *Centrifuge Box*—A centrifuge (International clinical centrifuge) with its housing removed (Fig. 4, *A*) is maintained at 37° in an insulated box (Fig. 4, *H*). In one of the shields (Fig. 4, *B*, of the size for a 50 ml. centrifuge tube) is to be placed a Lucite cylinder, the cross-sectional view of which is shown in Fig. 4, *I*. A counterweight in the other shield exactly balances the cylinder when the filled sample cell is inside the cylinder. The cylinder is 9 cm. long, with an inside diameter of 1.5 cm. and an outside diameter of 3 cm.; it serves as the insulation for the sample cell during centrifugation when the air temperature in the box might rise 0.5–1.0°. Greater rise of temperature is prevented by an air stream of constant temperature, *i.e.* 37°, flowing through the box at 200 liters per minute. The air-heating unit (Fig. 4, *E*) is similar to that unit described in the tonometer box section, except that the heating tape is 5.1 × 122 cm. and has a power rating of 125 watts at 110 volts. The temperature of the air stream is regulated by a thermoswitch (Fig. 4, *F*) and indicated by a thermometer inside a glass tube (Fig. 4, *C*). Another thermometer (not shown) which registers air temperature of the box is also read through the cover. Through the armhole in the Transite roof of the box, the Lucite cylinder holding the sample cell can be placed in the shield. A 25 watt tungsten light bulb (Fig. 4, *G*) maintains the box above room temperature and so the box temperature may be brought up to 37° relatively soon after the heated air stream is turned on. Under the above conditions the temperature of the sample cell inside the Lucite cylinder will not rise more than 0.1° during centrifugation.

Procedure

Sampling—The sampling syringe (5 or 10 ml.) is lightly lubricated with mineral oil, and a porcelain disk cut from a crucible is placed in the barrel. The syringe is then sterilized. Slightly more than enough sterile heparin solution (10 gm. per cent) to fill the dead space is drawn into the syringe. A hypodermic needle (No. 21), or a similar needle with its shaft elongated by a 20 cm. length of polyethylene tubing, is sealed onto the syringe with collodion to prevent air leakage. Air in the needle is replaced by the heparin solution from the syringe. The syringe is then placed in a Lucite jacket which serves as insulation during sampling. The assembled sampling device is warmed up to and maintained at 37°. Sterile technique is used up to this point. As soon as a sufficient amount (5 ml.) of blood has been drawn into the syringe, the latter is put in the tonometer box and the Lucite jacket is removed. About 0.01 volume (0.05 ml.) of fluoride solution (40 gm. per cent of sodium fluoride and 1.2 gm. per cent of potassium fluoride) is drawn through the needle into the syringe. The needle tip is stuck

into a rubber stopper. The fluoride solution and the blood are mixed thoroughly by allowing the porcelain disk to travel by gravity from one end to the other inside the syringe through inversions. The needle is removed from the syringe and replaced by a hypodermic needle (No. 19, 3 inches) with blunted tip. After the air is expelled from this needle, it is inserted downward into the sample cell, which has been previously filled with mercury. (This was accomplished by injecting mercury into the sample cell with the aid of a syringe and a long needle and then applying suction with a vacuum pump to remove the trapped air bubbles. The glass valve of the sample cell was kept open only during the evacuation with a thin stainless steel wire. The mercury-filled sample cell was capped with the greased ball joint and equilibrated at 37° before use.) Now the blood sample with inhibitor added is injected into the sample cell with the latter in an inverted position so that a thin stream of mercury flows between the ground opening of the ball joint and the shoulder of the needle. The displacement of mercury by blood permits anaerobic filling of the sample cell. When the cell has been completely filled, it is righted and the needle is slowly withdrawn while the needle space is replaced with blood. The ball joint is then rotated sidewise to seal off the sample cell, and the excess blood in the bore of the ball joint is removed by aspiration through the needle. The last trace of blood in the bore is wiped out with a wick. The sample cell is placed in a Lucite cylinder and immediately transferred to the centrifuge.

Centrifugation and Polarography—The sample cell is placed in the counterbalanced centrifuge through the armhole (Fig. 4, *D*) on the top of the box (maintained at 37° for 30 minutes prior to centrifugation) and centrifuged for 4 minutes at 2200 r.p.m. Deceleration is hastened by braking the spindle with a cork. The sample cell is removed from the centrifuge. The ball joint of the sample cell is removed with a stick inserted into the bore, and the sample cell is then dropped through the platinum wire loop (Fig. 2, *C*) into the 37° saturated potassium chloride bath. The wire loop serves to support the sample cell in an upright position, yet allows considerable flexibility for positioning of the cell with regard to the capillary dropping mercury electrode. The electrode should be wiped only shortly before its introduction into the sample cell; otherwise irregular dropping time will persist for several seconds until the plasma has wetted the tip of the capillary. While the potassium chloride bath is being positioned in the horizontal plane with one hand, the platform on which the bath rests is raised with the rack and pinion by the other until the socket joint of the sample cell touches the shoulder of the capillary electrode. At this point enough plasma is displaced from the sample cell to form a small pool in the socket joint, and the circuit is closed by dropping one end of the agar bridge into the plasma pool and the other into the salt bath.

As soon as the circuit is closed, the zero point of the polarograph is set near one end of the recording chart; then the potential is turned to exactly −0.4 volt, and the automatic voltage change is set in motion at the moment the recording pen is crossing a marker line on the chart. A reading is taken at −0.5 volt. The current range setting has been selected to give the highest possible reading of current on the chart within the voltage range −0.4 to −0.6. After each recording of the polarogram the potential is turned back from −0.6 to 0.0 volt and a reading of the zero point taken. The zero point reading is subtracted from the −0.5 volt reading, and the difference multiplied by the factor corresponding to the current range used yields the diffusion current in microamperes. Two more polarograms are recorded to serve as a check for the validity of the first. After the last polarogram the platform is lowered and the dropping mercury electrode rinsed and wiped clean. The tip of the electrode is then immersed and left in a beaker of distilled water between runs. The mercury leveling bulb is lowered only after the determination has been completed to a point below the side tube of the mercury column, and the flow of mercury gradually stops.

It is necessary to record the time of each step during the procedure so that the calibration run can be timed to simulate closely the sample run. The dropping time of the mercury can be readily calculated from the polarogram. Barometric pressure during the experiment is also recorded.

Calibration—Judging from the color of the blood, a rough estimation of its oxygen saturation may be made and, on the basis of saturation, the approximate range of the oxygen tension. Thus, 5, 10, or 15 per cent oxygen in a gas mixture of 5 per cent carbon dioxide and the remainder nitrogen is used to equilibrate with the blood sample. The equilibrating gas is first bubbled through distilled water in gas washers maintained at 37° in a water bath. The blood sample remaining in the sampling syringe is injected into the tonometer through its gas outlet hole; slightly more than that is required to fill the sample cell (2 ml.) is thus introduced with a long needle. After 1 hour of equilibration the blood in the tonometer is taken up in a 2 ml. syringe which has been lubricated with mineral oil and heated to 37°. The dead space of the syringe and needle has been filled with the gas mixture in equilibrium with the blood. The blood is then transferred into the sample cell and the remainder of the procedure is exactly as described for the original sample.

Calculation—The formula for the calculation of oxygen tension of the blood sample is as follows:

$$\text{O}_2 \text{ tension of sample in mm. Hg} = \text{O}_2 \text{ tension of standard gas} - \frac{\text{(sample microampere)} - \text{(standard microampere)}}{\text{slope of calibration curve}}$$

O_2 tension of the standard gas is obtained by multiplying the oxygen concentration of the standard gas in volumes per cent by the barometric pressure, corrected for water vapor pressure. Sample microampere and standard microampere refer to the diffusion current values from the polarograms. The determination of the slope of the calibration curve, (diffusion current)/(oxygen tension), will be described below.

EXPERIMENTAL

Experiments were carried out to ascertain (*a*) the linearity of diffusion current *versus* oxygen tension curve for pooled plasma, (*b*) the reproducibility of results when the proposed procedure is applied to blood samples, and (*c*) the slope (diffusion current)/(oxygen tension).

The linearity of the diffusion current versus oxygen tension curve described by previous investigators was confirmed with *pooled* plasma equilibrated with nitrogen containing 6.0, 10.3, 15.6, and 20.5 per cent O_2, respectively, and 5 per cent CO_2. A special tonometer and sample cell were constructed for this study which allow direct transfer of equilibrated plasma from the tonometer to the sample cell for polarography, but details of the apparatus will be omitted here. As will be seen later, the presence of cells in blood samples influences the actual diffusion current readings to the extent that no prediction can be made on the basis of the curve for pooled plasma as to the nature of the curve for blood. However, this study does indicate that the diffusion current is directly proportionate to the oxygen concentration of the plasma in the range examined, indicating the absence of complicating factors.

The reproducibility of results was established by two series of experiments. First, the diffusion current of a blood sample equilibrated with a gas of known oxygen tension was compared with the current of the plasma, obtained from the same equilibrated blood and reequilibrated under identical conditions. Second, comparison was made on blood samples divided into two portions and equilibrated with the same gas. Fluoride solution was added to the fresh venous blood samples before equilibration, and gases of different ranges of oxygen tensions were used for equilibration.

The results of the first series are reported in Table I; they demonstrate that no significant difference exists between the plasma and blood sample equilibrated with the same gas.

It was found that fresh blood kept at a given oxygen tension and later equilibrated under the same tension gave progressively lower values for diffusion current. This may explain the slightly lower diffusion current for the plasma sample. For this reason only duplicate runs in quick succession were made on blood samples in the second series of experiments (Table II). Since only one oxygen tension determination can be made on the fresh blood sample, there is no way to reduce the standard deviation of

the result by duplicate or triplicate analysis. However, the standard deviation of a single determination is not excessive for physiological studies.

The standard gases used for the equilibration were analyzed with the Scholander micro gas analyzer (2), and the oxygen content of the tanks

TABLE I

Comparison of Diffusion Current of Blood and Plasma Equilibrated with Same Gas

Oxygen tension	Diffusion current	
	Blood	Plasma
mm. Hg	*μa.*	*μa.*
37.5	1.432	1.410
	1.114	1.090
	1.136	1.104
	1.172	1.184
	1.182	1.182
77.0	2.092	2.070
	2.253	2.202
	1.980	1.962
	2.082	2.052
106	2.253	2.220
	2.781	2.709
	2.712	2.754
	2.541	2.565

TABLE II

Reproducibility of Results in Various Ranges of Oxygen Tension

Oxygen tension	No. of duplicate runs	Σ(per cent Δ)^2	Standard deviation	
			per cent	*mm. Hg*
mm. Hg				
30	8	123.8	2.8	0.8
75	9	52.1	1.8	1.4
140	9	35.2	1.4	2.0

Σ(per cent Δ)^2 = the sum of the squares of per cent difference between duplicate diffusion current determinations.

was found to be the same for at least 2 weeks. With a gas stream of constant oxygen content, there is no need to analyze the gas in the tonometer.

The constancy of the relationship between the *diffusion current and the oxygen tension* was determined within the range of application. With pooled plasma, there is a linear relation for these factors, as previously shown. Blood was equilibrated with gases of various oxygen tension and the diffusion current determined to discover whether the relation was sim-

ilarly linear for whole blood. Each blood sample was equilibrated with two gases, with a minimal time lag (1 hour) between determinations. The slopes, (diffusion current)/(oxygen tension), for the ranges 35 to 70, 70 to 100, 100 to 140 mm. of Hg were determined and tabulated (Table III), and from the range of values for the slope it seems that the diffusion current-oxygen tension curve is not a straight line for whole blood. Hence, one of the four standard gases with respective oxygen partial pressures of 35, 70, 100, and 140 mm. of Hg is needed for analyzing a blood sample of any normal physiological oxygen saturation. If one wishes to reduce the contribution of error due to the value of the slope, the standard gas oxygen tension should be as close as possible to the tension of the sample.

It is obvious that the value of the slope is characteristic of the dropping mercury electrode and the cell used. In case of breakage of either item, a new value for the slope must be determined under the identical conditions

TABLE III

Slopes of Diffusion Current versus Oxygen Tension Curve

Range of oxygen tension	Slope, 10^{-9} ampere per mm. Hg	Mean
mm. Hg		
35– 70	17.6, 18.2, 18.6, 18.8, 17.5	18.1
70–100	16.7, 15.7, 16.4, 16.8, 18.4	16.8
100–140	18.5, 15.2, 15.1, 17.4, 16.6, 16.0	16.5

employed for the analysis of blood samples. Since the electrode is protected by the flexible joints and the highly adaptable support of the sample cell, namely the platinum wire loop, the chances of its breakage are small.

DISCUSSION

Wiesinger's experience has been of great value in our work (1). We have eliminated interference by hemoglobin by keeping the sample free from hemolysis. With a few experiments in which hemolysis occurred, the slope of the calibration curve was unpredictable. Therefore the calibration for hemolyzed plasma suggested by Wiesinger would seem inaccurate, and it is preferable to discard hemolyzed samples.

There are two important periods in the procedure when the oxygen tension may drop: (*a*) from the time of sampling until the addition of the metabolic inhibitor, while the leucocytes continue to consume oxygen, and (*b*) during the separation of cells from plasma until the reading of the diffusion current, when the plasma oxygen is no longer maintained constant by equilibration with the cells. The second period is controlled by use of

our modified sample cell which shortens this crucial period (3). The total procedural time is 12 to 13 minutes.

With the use of heparin instead of oxalate as an anticoagulant, it is possible to add a greater concentration of fluoride to suppress the respiration of leucocytes of the blood sample without causing hemolyzing hypertonicity. In order to keep the volume of the fluoride solution minimal, so that the effect of the dissolved oxygen in the solution on the plasma oxygen tension would be negligible, a highly concentrated suspension of sodium fluoride was finally used. Since sodium fluoride goes into solution rather slowly, while potassium fluoride dissolves in water readily, the latter was added to effect quickly a high fluoride concentration in the blood sample. A final concentration of approximately 2 mmoles of potassium fluoride and 100 mmoles of sodium fluoride was found to be effective without causing any hemolysis. This required the addition of 1 volume of a mixture of 40 gm. per cent of sodium fluoride and 1.2 gm. per cent of potassium fluoride to 100 volumes of blood. It was observed that the blood invariably turns a considerably brighter red with the addition of fluorides, and this change of color indicates the distribution of the inhibitor in the sample. It was also found that the use of mercury to facilitate the mixing of the inhibitor invariably caused lower results; this phenomenon has been explained by Wiesinger on the basis of chemical reaction between mercury and chloride ions and casts doubts on the oxygen tension values in previous studies in which prolonged contact of the blood sample and mercury (and especially finely divided mercury) was permitted. To eliminate this source of error a porcelain disk was placed in the sampling syringe for mixing purposes prior to the displacement of air in the dead space with heparin solution.

It seemed feasible to set up a calibration curve so that the oxygen tension of a blood sample could be read off the curve once the diffusion current was determined. That has been the procedure used by all previous workers. Wiesinger attributed the variation of the absolute values of the diffusion current of human plasma to inadequate temperature control prior to the complete separation of erythrocytes from the plasma. This might very well be a major factor when one is dealing with blood samples. However, when plasma samples were obtained from the blood of human subjects and then equilibrated with gas of the same composition, the absolute diffusion currents were of considerably different values, as shown in Table I. These plasma samples are all free from detectable hemolysis, thus eliminating the contribution of hemoglobin on the diffusion current. We have further made the observation that the absolute value of the diffusion current decreased gradually in the course of several hours in spite of continuous equilibration of the blood with the same gas current. One is forced to conclude

that under the experimental conditions described in this report the assumption of a constant residual current for plasma of different individuals is not valid. In other words, even though the slope of the calibration curve may be assumed to be constant, the intercept of the curve is not constant for different blood samples. It follows then that for each blood sample a reference point must be obtained so that one may fix the intercept of the calibration curve. Further precision is gained by placing the reference point as close as possible to the point representing the sample. Therefore the proposed procedure seems to offer the simplest and yet most reliable method of calibration. When only the relative blood oxygen tension in the same subject during an experiment is of interest, one may omit the calibration for reference point.

SUMMARY

A polarographic procedure for the direct determination of oxygen tension on 5 ml. of saturated or unsaturated blood has been described. The precision of the method has been determined; the standard deviations of a single analysis for the oxygen tension ranges 30, 75, and 140 mm. of Hg are 0.8, 1.4, and 2.0 mm. of Hg, respectively.

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FURTHER STUDIES ON THE BIOSYNTHESIS OF ALDOLASE AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*

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When the biosynthesis of a protein occurs in the presence of labeled amino acids, the resultant pattern of labeling in the protein molecule may yield information about the intermediate steps in its synthesis. Anfinsen and coworkers (1, 2) have made extensive use of this approach and have been able to demonstrate that, in the synthesis of three proteins (ovalbumin, ribonuclease, and insulin) by tissue minces or slices, the residues of a given amino acid in different positions in the protein are labeled unequally. These investigators have interpreted their results to indicate a stepwise synthesis of protein involving intermediates, probably peptide in nature.

On the other hand, experiments with intact animals by Muir, Neuberger, and Perrone (3) on hemoglobin synthesis and by Askonas, Campbell, and Work (4) on the synthesis of milk proteins have shown uniform labeling of the residues of a given amino acid. A study of the incorporation of labeled amino acids into muscle aldolase and glyceraldehyde-3-phosphate dehydrogenase of the intact rabbit was reported previously (5). The data indicated that in each of these two proteins a given amino acid is equally labeled in whatever position it occurs in the protein molecule. This conclusion was based on the finding that when a number of labeled amino acids are administered to a rabbit the ratio of the average specific activity of all the residues of an amino acid in one protein to the average specific activity of all the residues of the same amino acid in the other protein is the same for all amino acids tested. An extension of this work to aldolase and phosphorylase by Heimberg and Velick (6) gave similar results.

In an attempt to find an explanation for the apparent lack of accord between the two sets of data obtained *in vitro* (1, 2) and *in vivo* (3–6), the possibility was considered that in the aldolase-triosephosphate dehydrogenase experiment the relatively long time interval between the administration of the labeled amino acids and the sacrifice of the animal might have permitted some type of isotope equilibration to occur. Such equilibration could have resulted in a gradual masking of an initial non-uniform labeling.

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Steinberg and Anfinsen (1) have presented evidence from experiments on ovalbumin *in vitro* which is in accord with this possibility.

In the present communication, data are presented on the labeling of aldolase and glyceraldehyde-3-phosphate dehydrogenase of rabbit muscle with a time interval of 30 minutes between the injection of the amino acids and the sacrifice of the animal. This interval is shorter than any hitherto used in such experiments, either *in vivo* or *in vitro*. It was chosen as a compromise between the desirability of an extremely short interval and the limited availability of the amounts of isotope needed for such an experiment. It was hoped to determine from the results whether, during the early stages of the incorporation, non-uniform labeling had occurred.

EXPERIMENTAL

Injection of Amino Acids—A 3.5 kilo rabbit, fed *ad libitum*, was injected in the ear vein with a solution containing glycine-1-C^{14}, DL-alanine-1-C^{14}, DL-lysine-2-C^{14}, L-methionine-S^{35}, DL-phenylalanine-3-C^{14}, and $NaHC^{14}O_3$. The $NaHC^{14}O_3$ was administered in order to increase the labeling of the dicarboxylic amino acids, which was very low in the 38 hour experiment.

Isolation of Enzymes—The animal was killed 30 minutes after the injection, and aldolase and triosephosphate dehydrogenase were crystallized from extracts of the skeletal muscle by the method of Cori and coworkers (7). The presence of ethylenediaminetetraacetic acid (EDTA) has been shown to prevent loss of activity of the dehydrogenase on standing by inhibiting oxidation of its sulfhydryl groups (8). In this laboratory the results of several trial runs prior to the radioactive run indicated that the use of EDTA in the isolation procedure resulted in an increase in the final yield of this enzyme. Accordingly, EDTA (neutralized) was used in the isolation and purification procedures, and its concentration at all steps was maintained at 60 γ per ml. The enzymes were recrystallized six times. The final products met the criteria of purity described previously (5). The final yields were as follows: aldolase 410 mg., glyceraldehyde-3-phosphate dehydrogenase 1.62 gm.

Isolation of Amino Acids—The purified enzymes were dialyzed against mercaptoethanol, precipitated and washed with trichloroacetic acid, and further washed with alcohol and ether. They were then hydrolyzed and the individual amino acids isolated by ion exchange chromatography (9), followed by preparative paper chromatography. A fraction containing serine and the dicarboxylic amino acids was lost. Radioactivity was measured by plating on copper planchets and counting in a windowless flow counter whose background count was 13 c.p.m. Details of these procedures as well as the criteria of amino acid purity have been described previously (5).

Results

The results of this experiment are reported in Table I, and for comparison the data of the 38 hour experiment (5) are included. As expected, the specific activities of the amino acids are considerably lower in the short term experiment. The differences would be even greater if the data were

TABLE I

Specific Radioactivities of Amino Acids Isolated from Aldolase and Glyceraldehyde-3-phosphate Dehydrogenase

Specific activity in counts per minute per micromole.

Amino acid	Substance counted	30 min. experiment			38 hr. experiment (Simpson and Velick (5))		
		Aldolase	Dehydrogenase	Ratio, aldolase / dehydrogenase	Aldolase	Dehydrogenase	Ratio, aldolase / dehydrogenase
Lysine	Lysine HCl	20.2	12.4	1.63	20.2	11.4	1.77
Methionine	Benzidine sulfate	14.8	9.75	1.52	108	59.5	1.82
Phenylalanine	Phenylalanine	5.40	3.50	1.54	83.2	43.5	1.91
Tyrosine	Tyrosine	1.88	1.39	1.35	37.8	21.4	1.77
Glycine	$BaCO_3$ (carboxyl C)	1.09	0.710	1.54	16.4	9.92	1.65
Alanine	" " "	0.916	0.677	1.35	4.29	2.31	1.86
Serine	" " "				9.97	5.22	1.91
Glutamic acid	" " "				0.756	0.422	1.79
Mean				1.49			1.81

Injected doses and specific activities were as follows: *30 minute experiment*, 0.2 mc. of glycine-1-C^{14} (1.3 mc. per mmole), 0.2 mc. of DL-alanine-1-C^{14} (1.1 mc. per mmole), 0.2 mc. of DL-lysine-2-C^{14} (0.37 mc. per mmole), 0.5 mc. of L-methionine-S^{35} (4.5 mc. per mmole), DL-phenylalanine (0.78 mc. per mmole), $NaHCO_3$-C^{14} (7.1 mc. per mmole); *38 hour experiment*, 0.1 mc. each of the above amino acids possessing specific activities of 0.5, 0.85, 0.43, 2.0, and 0.5 mc. per mmole, respectively. No $NaHCO_3$ was used.

corrected for the different amounts of radioactivity administered in the two experiments. The extent of the decrease, however, is not the same for all amino acids. Variation between animals with regard to factors such as composition of the free amino acid pools and rates of change of the specific activities of the amino acids in these pools could account for such differences.

In Table I, the results are expressed as a series of ratios of the specific activity of an amino acid in aldolase to the specific activity of the same amino acid in the dehydrogenase. As was previously found in the 38 hour

experiment, the ratios are very close to one another. The standard deviation of a single ratio is about ±8 per cent; the standard error is about ±3 per cent. Since the plating and counting errors may be estimated to be about ±4 per cent (5), the small additional error in excess of 4 per cent could easily be the result of the presence of slight amounts of impurities. Until much larger differences between ratios can be demonstrated, it appears reasonable to regard these ratios as being equal.

It may also be noted in Table I that the mean ratios in the two experiments are not the same (1.49 and 1.81). This difference, however, cannot be a result of the two different time intervals used, since in a similar study on aldolase and phosphorylase (6) the ratios for the different amino acids remained equal, although each labeled amino acid was injected into the same rabbit at a different interval prior to the sacrifice of the animal. Inasmuch as different animals were employed in the two aldolase-dehydrogenase experiments, it seems more reasonable to ascribe the different mean ratios obtained in the two animals to biological variation.

DISCUSSION

From the equality of the specific activity ratios, it may be inferred that in a given protein each residue of a particular amino acid possesses the same specific activity, since non-uniform labeling would be reflected in unequal ratios; the arguments for this view-point have been presented previously (5). However, conclusive evidence for uniform or non-uniform labeling can only come from experiments in which the specific activities of the residues of a given amino acid in separated fragments of the protein are compared. Such experiments with glyceraldehyde-3-phosphate dehydrogenase are now in progress.

Perhaps the simplest interpretation of uniform labeling is that the cellular apparatus for protein synthesis uses free amino acids directly, since the immediate precursors of all the residues of a given amino acid in a uniformly labeled protein must have the same specific activity. However, in order to interpret the results as completely excluding peptide intermediates, the assumption must be made that the failure to find evidence for them in these experiments was not a result of isotope equilibration.

Evidence for the occurrence of equilibration reactions exists. Steinberg and Anfinsen (1), in studies on the incorporation of labeled amino acids into ovalbumin by minced oviduct, have demonstrated that the pattern of labeling changes with time. This protein, which showed appreciable differences in the specific activities of aspartic acid and of glutamic acid residues after 1 hour, appeared to become more equally labeled during the succeeding 7 hours of the incubation, although complete uniformity of labeling was far from being reached in this time. If, in the biosynthesis

of the muscle enzymes described in the present paper, there occurs a transition from non-uniform labeling to uniform labeling, this must have occurred in less than 30 minutes, a time much shorter than was found for such a transition in the experiments of Anfinsen and Steinberg. Furthermore, since the rate of protein synthesis is relatively slow in muscle, the 30 minute experiment provides favorable conditions for observing such an incomplete transition. It may be concluded, therefore, that, whatever the reactions leading to the slow equilibration in the minced oviduct system, the short time interval employed in the present experiment makes this type of behavior unlikely in the labeling of the muscle proteins.

It is difficult, however, to rule out the occurrence of certain very rapid reactions leading to isotope equilibration. One extreme possibility that cannot be discounted would involve rapidly reversible reactions between amino acids and peptides with a slow non-reversible step between peptides and protein. These reactions could lead to uniform labeling of the protein. No direct evidence exists for or against such a process.

SUMMARY

The incorporation of labeled amino acids into muscle aldolase and glyceraldehyde-3-phosphate dehydrogenase was studied in the intact rabbit with intervals of 30 minutes between the injection of the amino acids and the sacrifice of the animal. This interval is shorter than any hitherto used in such experiments, either *in vivo* or *in vitro*. The pattern of labeling indicated that in each of these two proteins a given amino acid is equally labeled in whatever position it occurs in the protein molecule. The significance of these findings with respect to proposed mechanisms of protein synthesis is discussed.

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THE PREPARATION AND CHARACTERIZATION OF RIBONUCLEIC ACIDS FROM YEAST*

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Considerable variation in composition occurs among samples of ribonucleic acids from yeast. These variations are due in part to the procedures that are employed for isolation and subsequent purification. A recent study by Loring, Fairley, and Seagran (1) notes that variations may be due to (*a*) the occurrence of different ribonucleic acids in the particulate components of cells, (*b*) partial enzymatic degradation, and (*c*) partial chemical degradation. All methods recently published for isolation have sought to avoid partial chemical degradation by complete abandonment of earlier drastic procedures which incorporated the use of extremes in pH and prolonged treatment by heat. However, in discarding drastic methods and accepting milder methods which involve lengthy extractions at physiological pH, the way has been opened for enzymatic degradation to occur in a considerable degree.

That enzymatic degradation may occur during procedures to isolate ribonucleic acids from tissues was first demonstrated by Bacher and Allen (2). Volkin and Carter (3), by the use of guanidine hydrochloride, and Kay and Dounce (4), by the use of sodium dodecyl sulfate, have developed procedures to minimize enzymatic degradation. However, neither of the procedures effects the complete inhibition of crystalline ribonuclease from beef pancreas.[1] The rapidity of destruction of ribonucleic acids in yeast is shown by a recent report of Bourdet and Mandel (5) wherein autolysis for 10 minutes at 50° destroys 93 per cent of the nucleic acids originally present.

The procedure to be described was designed specifically to eliminate enzymatic degradation as a factor during isolation. The ribonucleic acids that have been isolated by the use of this procedure differ significantly in certain properties from samples described heretofore.

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[1] Unpublished observations.

Preparation

Materials and Reagents—Bakers' yeast (*Saccharomyces cerevisiae*) was supplied directly from the filter press by the Consumers Yeast Company, Oakland, California.

Sodium dodecyl sulfate was prepared by the extraction of 300 gm. batches of Duponol C with 7 liters of boiling ethanol. The extract was filtered while hot through a Celite pad on a Büchner funnel. Crystallization from the filtrate at 0° yielded 178 gm. of ether-dried product. The sulfur content of the product was 9.8 per cent as compared with 11.1 per cent for sodium dodecyl sulfate. Difficulty was encountered in the attempted use of several products sold as sodium dodecyl sulfate.

Step 1. Extraction of Nucleic Acids—A 4 liter beaker is fitted with a top plate that contains a narrow slot through which a motor-driven stirring rod and a thermometer can be introduced. 500 ml. of a solution which contains 2 per cent sodium dodecyl sulfate, 4.5 per cent ethanol, 0.0125 M NaH_2PO_4, and 0.0125 M Na_2HPO_4 are placed in the beaker. A Fisher burner is used to bring the solution to boiling with constant stirring. The top plate is removed, and, with constant stirring, 150 gm. of yeast cut into very fine pieces are added as one batch to the boiling solution. The top plate is immediately replaced to reduce loss of water by evaporation. The temperature after the addition of yeast ranges from 83–87°. Heating is continued with stirring for 1 minute, after which the beaker and contents are transferred to a boiling water bath for 2 minutes in order to avoid excessive foaming. At the time of transfer to the water bath the temperature of the solution ranges from 92–94°. After 2 minutes in the water bath the contents of the beaker are poured into a 2 liter beaker which is immersed in a solid carbon dioxide-Cellosolve freezing mixture. The contents of the beaker are stirred manually for 4 to 8 minutes until the temperature drops to approximately 4°. The mixture is centrifuged at 2000 r.p.m. for 30 minutes at 0°.

Comments—Experiments were conducted to study the relationship between the concentration of detergent and time of heating necessary to effect maximal extraction of ribonucleic acids from the yeast. Ribonucleic acids were determined by modifications of the method of Scott and Fraccastoro (6). Sodium dodecyl sulfate concentrations were varied from 0.5 to 5.0 per cent. The heating period was varied from 2 to 10 minutes. With 2 per cent concentration of sodium dodecyl sulfate and a 3 minute heating period, 90 per cent of the ribose nucleic acids present in the yeast were found to be present in the supernatant liquid. No advantage was found by increasing either the time of heating or the concentration of sodium dodecyl sulfate. The addition of the phosphate buffer maintained the pH of the supernatant fluid between 6.5 and 6.7.

Experiments which were conducted to test the effect of heating and sodium dodecyl sulfate concentration on the activity of crystalline ribonuclease from beef pancreas showed complete inactivation by 0.5 per cent sodium dodecyl sulfate at pH 7.0 and 3 minutes heating.

Step 2. Preparation of Crude Ribonucleic Acids—The supernatant fluid from Step 1 is poured with stirring into 2 volumes of cold ethanol. The precipitate is collected by centrifugation at 2000 r.p.m. in the cold for approximately 15 minutes. The collected precipitate is then washed with two 150 ml. portions of cold 67 per cent ethanol. In washing it is advisable to homogenize the precipitate with a glass rod and add the wash liquid slowly. 5 to 10 drops of a 2 N solution of sodium chloride are added to insure flocculation and recovery. 15 to 20 minutes centrifugation at 2000 r.p.m. are sufficient for each step in the washing. The crude nucleic acids are suspended in 80 per cent ethanol and permitted to stand overnight in the cold room to aid in the removal of protein.

Comments—The precipitate, if collected and analyzed at this point, contains over 80 per cent of the ribonucleic acids that were present in the original yeast. The precipitate itself is 60 to 65 per cent ribonucleic acids. Analysis of the precipitate for ribonuclease activity yielded negative results.

Step 3. Purification of Ribonucleic Acids—The crude nucleic acids from Step 2 are collected by centrifugation and dissolved in 130 to 180 ml. of water. The solution is turbid, with a pH of approximately 8. The solution is immediately neutralized (pH 7.0) by the addition of 1 N acetic acid. Turbidity is removed by centrifugation for 30 minutes at 20,000 r.p.m. at 0° in a Spinco preparative centrifuge. A certain amount of floating material is not removable at this point. Solid sodium chloride is added to the supernatant fluid to bring the final concentration to 1 M. Upon standing at 0°, the turbidity increases slightly and the viscosity increases markedly. In about 30 minutes a gel separates. The supernatant fluid, which is yellow and contains a small amount of floating material, is separated from the gel by centrifugation at 2000 r.p.m. for 1 hour. The supernatant liquid is discarded. The gel is washed successively with three 150 ml. portions of 67 per cent ethanol, to each of which has been added 1 ml. of a 2 M solution of sodium chloride. The washed precipitate is dissolved by the slow addition of water with continuous stirring. The pH of the solution is 7. It is dialyzed for 36 hours at 4° against frequent changes of distilled water. Turbidity is removed, first by filtration through Celite and finally through a D-7 Steriflo asbestos pad (F. R. Hormann and Company, Newark, New Jersey). The clear, colorless solution is lyophilized to yield a white solid. The yield is 1.4 gm., which is equivalent to 60 to 70 per cent of that originally present in the yeast.

Comments—Many procedures for the preparation of deoxyribonucleic acids (7–10) and for the preparation of ribonucleic acids (4, 11, 12) employ 1 M sodium chloride for the separation of denatured proteins from nucleic acids. The proteins are expected to sediment, while the nucleic acids remain in solution. Much to our surprise it was found that 1 M sodium chloride as employed in Step 3 brought about the precipitation of 90 per cent of the ribonucleic acid present in the solution. According to our knowledge the precipitation of ribonucleic acids at 0° by addition of sodium chloride to 1 M concentration has not been reported previously. A study of the effect of sodium chloride concentration from 0.15 to 2.15 M showed that maximal precipitation of ribonucleic acids occurred in the range from approximately 1 to 2 M sodium chloride. No trend in the amounts of nonnucleic acid substances that are precipitated could be demonstrated. Contaminating proteins and deoxyribonucleic acids are mainly soluble in 1 M sodium chloride. Deoxyribonucleic acids as contaminants were measured by the method of Stumpf (13). The ribonucleic acids from Step 3 contain less than 0.5 per cent deoxyribonucleic acids. Semiquantitative estimation of contamination by proteins was performed by the use of the bromophenol blue staining technique as described by Kunkel and Tiselius (14). Less than 2 per cent protein contamination was found. Since further reduction of the protein contamination involves the use of time-consuming procedures (15) in which phosphodiester bonds may be broken, it was felt advisable to charaeterize the sample without further purification.

Properties

All data are calculated on the basis of the dry weight obtained by drying to constant weight in a vacuum oven at 110°.

Phosphorus—Total phosphorus was determined by a modification of the procedure by Griswold *et al.* (16).

Mononucleotide Composition—Alkaline hydrolysis and chromatography were carried out as described by Smith and Allen (17). Mononucleotide areas were eluted by the use of the method of Hotchkiss (18). Spectrophotometric data were converted to moles of nucleotide by use of the extinction coefficients of Cohn (19). Recoveries of mononucleotides accounted for 95 per cent of the total phosphorus and 95 per cent of the total ultraviolet absorption of the alkaline hydrolysate at 260 mμ. The polynucleotides which were present in the alkaline hydrolysate and which were evident on chromatograms were estimated to be of the same order of magnitude (approximately 3 per cent) as those found by Smith and Allen (17).

Mononucleotides Liberated by Ribonuclease Action—Hydrolysis by ribonuclease was accomplished by the serial addition of 10 μl. portions of a 10 per cent solution of ribonuclease (Armour, lot 38959) at 0, 2, and 6 hours to 500 μl. of a 4.7 per cent aqueous solution of the ribonucleate. Hydrol-

ysis was complete in 8 hours. Chromatography, elution, and spectrophotometry were carried out as described in the foregoing paragraph.

TABLE I

Chemical and Physical Properties of Ribonucleate from Yeast in Comparison with Data of Other Investigators

The figures in parentheses are bibliographic citations.

	Present study	Comparative data
Phosphorus, % *dry weight*	8.2	
Adenylic acid, *moles per 100 moles*	25	26 (21), 22 (21), 29 (22), 27 (23), 21 (24)
Guanylic " " " *100* "	28	29 (21), 34 (21), 24 (22), 26 (23), 32 (24)
Cytidylic " " " *100* "	20	21 (21), 23 (21), 20 (22), 21 (23), 25 (24)
Uridylic " " " *100* "	27	24 (21), 22 (21), 28 (22), 24 (23), 23 (24)
Polynucleotides, % *of total*	3	
Cytidylic acid liberated by ribonuclease, % *of total*	48	29 (22), 57 (24)
Uridylic acid liberated by ribonuclease, % *of total*	50	31 (22), 57 (24)
Buffer capacity pH 6.0–8.0, *mole* OH^- *per mole P*	0.032	0.085* (20), 0.03 (25)
Acidic groups liberated by ribonuclease		
(*a*) *mole per mole P*	0.45	0.28 (26), 0.34* (20)
(*b*) " " " *total mononucleotide*	0.48	
(*c*) " " " " *pyrimidine*	1.0	0.72–0.92 (27)
(*d*) *moles per mole liberated pyrimidine mononucleotide*	2.1	1.6 (27)
$E^{1\%}_{1\,cm.}$ 260 mμ, 0.01 M HCl	208	
" after alkaline hydrolysis, 260 mμ, 0.01 M HCl	284	
$E^{1\%}_{1\,cm.}$ after ribonuclease action, 260 mμ, 0.01 M HC	269	
Sedimentation, $s_{20,w}$	2.8–4.0	1.9* (28), 1.1–2.3* (29), 2.1* (30), 1.6–4.4 (31)
" "	7.0–9.5	
Intrinsic viscosity, $[\eta]_Y$	70	7.8*† (30), 13*† (32), 17–30 (33)

* Commercial ribonucleic acid.

† Reduced viscosities.

Titrimetric Data—The proportion of secondary phosphoryl dissociations was determined by titration of a 0.38 per cent solution of the ribonucleate in 0.10 N sodium chloride with 0.01 N solution of sodium hydroxide. Precautions for exclusion of carbon dioxide were observed. The titer between

pH 6.0 and 8.0 was doubled and considered to represent the maximal amount of secondary phosphoryl groups (20).

Acidic groups which were liberated as a result of ribonuclease action were determined by titration to pH 7.5 of a suitable control and the enzymatic digest in 0.10 N sodium chloride.

Sedimentation and Viscosity—Sedimentation measurements at 25° were made in a Spinco ultracentrifuge. Viscosity measurements at 30° were made in Ostwald viscosimeters. All sedimentation studies were performed on a 1 per cent solution of nucleate in 0.2 M sodium chloride which contained 0.05 M sodium phosphate buffer at pH 6.8. Viscosity measurements were made in the same solvent.

The data which were obtained by the foregoing analytical procedures together with certain of the data for specimens of ribonucleic acids from yeast from other investigations are given for comparison in Table I.

DISCUSSION

Partial enzymatic degradation during isolation has been eliminated as a factor in the present method. As far as is known, no one of the steps in the isolation could bring about the rupture of phosphodiester bonds. It seems reasonable then to assume the absence of polynucleotide fragments in the sample.

In the procedure 60 to 70 per cent of the ribonucleic acids which are present in the yeast are isolated. Accumulative losses of 30 to 40 per cent of the total are accounted for at each step of the procedure. Unless the losses should prove to contain a higher proportion of one or several species, the preparation can be considered as representative of the distribution of species of ribonucleic acids in yeast.

The composition of ribonucleic acids in terms of mononucleotide constituents can be assumed to be known only for mixtures of the nucleic acids and their degradation products. Until the mononucleotide composition is explicitly known for a given ribonucleic acid molecule, such information, while useful, is not definitive. Samples of ribonucleic acids which contain a high percentage of fragments as a result of partial enzymatic degradation by ribonuclease may contain a low proportion of cytidylic and uridylic acids. The proportion of uridylic acid in the present sample is high, but that of cytidylic acid shows little deviation from data of previous investigators.

Ribonuclease liberates part of the pyrimidine constituents of ribonucleic acids as free mononucleotides; the remainder of the pyrimidines appears as end-groups of purine-containing polynucleotides. These end-groups have thus far been identified as 3′-pyrimidine nucleotides which are esterified through their 5′ positions to adjacent purine nucleotides (22, 27). The liberation of a pyrimidine nucleotide, whether as a free mononucleotide or

as the end-group of a polynucleotide, is accompanied by the liberation of a secondary phosphoryl group. If it is assumed that no pyrimidine nucleoside is linked in such a way that ribonuclease cannot release a secondary phosphoryl dissociation from at least one of the phosphate groups esterified with it, then the secondary phosphoryl groups liberated should equal the pyrimidine content. This result is obtained for the present sample. Less than an equivalent amount of secondary phosphoryl dissociations (24) would be liberated if (*a*) the terminal phosphate groups of the undegraded nucleic acid had been esterified at the 2′ or 3′ position of pyrimidine nucleoside residues, (*b*) enzymatic and chemical degradation during isolation of the sample had exposed an additional proportion of the pyrimidine content as chain endings of this same type, and (*c*) some pyrimidine constituents are linked within chains in such a manner that ribonuclease cannot act on either side.

The possibility that some of the acidic groups liberated by ribonuclease show primary phosphoryl dissociations (34) is not excluded. These groups would be completely titrated under the conditions employed and would be called secondary phosphoryl dissociations. The titration curves before and after ribonuclease action permit an estimate to be made of the number of the released acidic groups accounted for as secondary phosphoryl dissociations. Such estimations show that 9.6 per cent of the new acidic groups are not found as secondary phosphate dissociations of pK 6.0. However, this figure reduces to 4.5 per cent if a pK of 5.9 is taken.

An accurate interpretation of the buffer capacity of the isolated ribonucleic acids between pH 6.0 and 8.0 in terms of the proportion of secondary phosphoryl groups requires information concerning the ionization of other groups in the ribonucleic acids in this region. Such data are known only for mononucleotides. If all of the buffer capacity in this region is due to the dissociation of a secondary phosphoryl group with a pK of 6.0, then the sample contains one secondary phosphoryl group per 16 phosphorus atoms. However, it is possible that other dissociating groups are partially titrated in the region of pH 6 to 8. In this case there would be less than one secondary phosphoryl group per 16 phosphorus atoms. These data are comparable to those of Gulland *et al.* (35) for their preparation of deoxyribonucleic acids from thymus and mark the first time that such a similarity between a sample of deoxyribonucleic acids and ribonucleic acids from yeast has been noted in this pH region.

Experience in this laboratory with the neutral sodium salts of other preparations of ribonucleic acids had shown such samples to be readily soluble in water. The preparation that is obtained in this procedure is a white, tough, fibrous material. When placed in water, the fibrous mass imbibes water to form a gel which, regardless of agitation to promote solution, is only slowly soluble. In the studies on viscosity, the results were

found to vary according to the amount of mechanical action which was employed to effect the solution of the sample as well as to the aging of the solutions. Different initial viscosities are obtained if solutions are compared as prepared (*a*) by slowly dissolving the sample with minimal mechanical action, (*b*) by grinding the preparation during solution with a Teflon homogenizer, (*c*) by grinding during solution with a glass homogenizer, or (*d*) by permitting the mixture to stand at 70° for 5 minutes. Regardless of the different initial viscosities, the relative viscosities of solutions in 0.2 M sodium chloride and 0.05 M sodium phosphate buffer at pH 6.8 decreased rapidly during the first 5 to 6 hours. Even after 16 hours lower viscosities were obtained when solutions were prepared by mechanical grinding of the sample into the solvent. The intrinsic viscosity of a solution which was prepared without mechanical grinding and which was aged for 11 hours at 30° was considerably higher than that reported for any previous sample of ribonucleic acids from yeast. It may be worthy of note that viscous solutions of ribonucleic acids from yeast and other sources (11, 33, 36) have been observed in cases in which heat was employed during the isolation procedures.

Sedimentation in an ultracentrifuge yielded two boundaries. Freshly dissolved samples contained predominantly the slower component. Samples which had been stored for 1 to 2 days in the refrigerator were mainly the faster component. The formation of two boundaries together with the observation that a gradual increase in the area of the faster boundary took place at the expense of the area under the slower boundary cannot be explained at present. The sample under investigation could contain many molecular species of ribonucleic acids in addition to some residual protein and at least one extraneous ion (0.3 per cent magnesium) capable of effecting the formation of complexes. Even in the event of a single molecular species it would seem that the interpretation of sedimentation data for ribonucleic acids must await further studies on the effects of pH (3), buffer species (31), and of nucleate concentration (37).

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SUMMARY

The isolation of 60 to 70 per cent of the ribonucleic acids of bakers' yeast by a procedure in which nuclease action is prevented and nucleic acids are extracted by the use of sodium dodecyl sulfate at 83–94° is described. During this procedure, removal of contaminating proteins and deoxyribonucleic acids is accomplished through the newly found precipitation of ribonucleic acids from 1 M sodium chloride solution at 0°.

The isolated sample has fewer secondary phosphoryl dissociations and a higher percentage of phosphodiester bonds which are susceptible to hydrolysis by ribonuclease than previous samples had. Other properties are described and discussed.

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URIDINE DIPHOSPHATE ACETYLGALACTOSAMINE IN LIVER*

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Several uridine diphosphate sugar compounds have been recently isolated; one of them, UDPG[1] (1), acts as coenzyme in the transformation of galactose-1-phosphate into glucose-1-phosphate and as glucose donor in the synthesis of trehalose phosphate (2), sucrose (3, 4), and sucrose phosphate (5). Similar compounds in which the glucose residue is replaced by galactose (6), acetylglucosamine (7), an amino sugar derivative plus amino acids (8), or glucuronic acid (9) have been described.

UDPAG was first isolated from yeast (7) and more recently has been detected in rat liver by Smith and Mills (10) and by Hurlbert and Potter (11). The present paper[2] reports its isolation from bovine liver. However, the product obtained was not a pure substance, but a mixture of UDPAG with a hitherto undescribed compound which appears to be UDP acetylgalactosamine.

Isolation of Nucleotides—An alcoholic extract from liver was run through an anion exchange resin (Dowex 2) and eluted with solutions of increasing chloride concentration according to the simplified procedure of Cabib *et al.* (7). The uridine nucleotides obtained by this method were concentrated by adsorption on charcoal, followed by elution with 15 per cent pyridine in water. The pyridine was extracted with chloroform, and the aqueous solution was concentrated by vacuum distillation. Further purification was obtained by paper chromatography.

Properties of the Substance—The absorption spectra of UDPAG from liver and yeast were found to be identical. They corresponded to that of uridine and showed the same changes with pH.

As in yeast UDPAG, there were two phosphate groups per molecule of

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[1] The following abbreviations are used: UDP for uridine diphosphate, UDPG for uridine diphosphate glucose, UMP for uridine monophosphate, UMP-5′ for uridine-5′-phosphate, UDPAG for uridine diphosphate acetylglucosamine.

[2] A preliminary note has been published (12).

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uridine. One of them is acid-labile and can be hydrolyzed in 30 minutes with 1 N acid at 100°. Comparative hydrolysis curves of UDPAG from liver and yeast are presented in Fig. 1. After acid hydrolysis, uridine-5′-

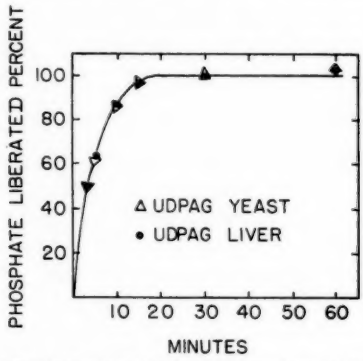

FIG. 1. Comparative hydrolysis of the labile phosphate of UDPAG from yeast and liver in 1 N acid at 100°.

TABLE I

Paper Chromatography of Nucleotides Obtained by Acid Hydrolysis of UDPAG from Liver

Substance	$R_{adenosine}$ values of ultraviolet-absorbing spots	
	Ethanol-ammonium acetate	
	pH 7.5	pH 3.8
Yeast UDPAG	0.56	0.57
" " + liver UDPAG	0.55	0.57
Liver UDPAG	0.55	0.56
UDP	0.16	0.45
Liver UDPAG heated 15 min. in 0.01 N acid at 100°	0.16	0.45
UMP-5′	0.29	0.75
Liver UDPAG heated 30 min. in 1 N acid at 100°	0.29	0.75

pyrophosphate and uridine-5′-monophosphate were identified as breakdown products of UDPAG of liver by paper chromatography in two different solvents (Table I). The standards for comparison were UMP-5′ and UDP obtained from UDPG or from UDPAG of yeast.

Identification of Carbohydrate Moiety—Mild acid hydrolysis of UDPAG from liver was found to liberate substances which give a positive Morgan-

Elson reaction (13). These substances were submitted to paper chromatography on borate-treated paper with butanol-pyridine-water as solvent (14), followed by spraying with benzidine (15) or with the modified Elson-Morgan reagent (16). Two spots were obtained having the same $R_{glucose}$ values and color as acetylglucosamine and acetylgalactosamine (Table II). On the other hand, with the yeast fraction only one spot was detected, corresponding to acetylglucosamine.

TABLE II

Paper Chromatography of Sugar Moiety from UDPAG

Solvent, butanol-pyridine-water (24 hours).

Substance	$R_{glucose}$ values of spots	
	Untreated paper	Borate-buffered paper
Acetylgalactosamine	1.25	1.43
Acetylglucosamine	1.34	1.68
Hydrolyzed liver UDPAG	1.26, 1.34	1.43, 1.68
" yeast "	1.33	1.68

TABLE III

Paper Chromatography of Deacetylated Sugars from Liver UDPAG

Solvent, butanol-pyridine-water (38 hours).

Substance	$R_{glucose}$ of spots
Galactosamine	0.64
Glucosamine	0.72
Deacetylated acetylglucosamine	0.71
" acetylgalactosamine	0.62
" sugars from liver UDPAG	0.64, 0.72
" " " yeast "	0.72

When the sugars from UDPAG from liver were submitted to hydrolysis under conditions which lead to complete deacetylation, they gave a positive Elson-Morgan-Blix reaction (17) for hexosamines. Paper chromatography of the hydrolysate showed two spots having the same $R_{glucose}$ values as glucosamine and galactosamine (Table III). Only the spot corresponding to glucosamine was obtained after treating UDPAG from yeast in the same manner (Table III). Furthermore, when the hexosamines obtained by deacetylation of the free sugars were treated with ninhydrin, according to a modification of the technique described by Gardell *et al.* (18), the corresponding pentoses, arabinose and lyxose, were obtained (Table IV). The hexosamine from yeast UDPAG gave only arabinose (Table IV).

The ratio uridine-total phosphate-acetylhexosamine was found to be 1.02:2:0.7 for liver UDPAG (total phosphate taken as 2). The low color value for acetylhexosamine can be explained by the fact that acetylgalactosamine gives only 35 per cent of the color obtained with acetylglucosamine in the modified Morgan-Elson test.[3]

TABLE IV

Paper Chromatography of Pentoses from Hexosamines of Liver UDPAG

The pentoses were obtained by treatment with ninhydrin, as described in the text. Solvent, butanol-pyridine-water (24 hours).

Substance	$R_{glucose}$ of spots
Arabinose	1.13
Lyxose	1.40
Pentose from glucosamine	1.12
" " galactosamine	1.39
" " hexosamines of liver UDPAG	1.12, 1.39
" " " " yeast "	1.12

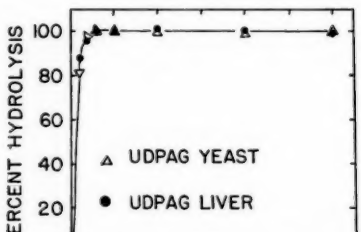

FIG. 2. Comparative liberation of acetylhexosamine from UDPAG from yeast and liver heated at pH 2, 100°. Acetylhexosamine was estimated by the modified Morgan-Elson method (see foot-note 3).

On the other hand, the ratio found by estimating the hexosamines after deacetylation was 1.02:2:1, as would be expected, since glucosamine and galactosamine yield the same intensity of color in the Elson-Morgan-Blix test (17). These two sets of results can be used to calculate the composition of the mixture, which turns out to be 75 per cent acetylglucosamine and 25 per cent acetylgalactosamine.

Quantitative estimation of the acetylhexosamines was also carried out

[3] Reissig, J. L., Strominger, J. L., and Leloir, L. F., in preparation.

by extracting the color developed with the "benzidine reagent" (15) after paper chromatography, with, in general, the procedure described by Aronson (19). The values obtained corresponded to about 67 per cent of acetylglucosamine and 33 per cent of acetylgalactosamine.

Fig. 2 presents a curve showing how the acetylhexosamine residue is liberated by acid. There is no observable difference between UDPAG from liver and yeast; so that the rate of release of the acetylgalactosamine moiety must be nearly the same as that of acetylglucosamine.

DISCUSSION

The acetylgalactosamine-containing compound could not be separated from UDP acetylglucosamine despite the fact that many procedures were tried, such as paper chromatography or electrophoresis with borate buffer or gradient elution of Dowex 1 columns with various solvents. This is an indication that both substances are closely related in structure, and, since no other substances besides UDP or UMP could be detected as degradation products, it can be concluded that acetylgalactosamine is linked to UDP. Other facts support this interpretation: the absorption spectrum is typical for uridine and the analytical data give the correct ratio for uridine-phosphate-(acetylglucosamine + acetylgalactosamine). Moreover, mild acid hydrolysis leads to the liberation of both acetylhexosamines at the same rate. These facts make it highly improbable that the acetylgalactosamine residue is joined to some substance different from UDP. It is likely that any other component representing 20 or 30 per cent of the total would have been detected; hence it can be concluded that bovine liver contains UDP acetylgalactosamine.

It has been found (11) that liver contains both UDP glucose and UDP galactose. Moreover, an enzyme is present in galactose-adapted yeasts which catalyzes the interconversion of these substances (6). Such an enzyme is probably present in liver, and it seemed likely that liver enzymes should catalyze the interconversion of the UDP acetylhexosamines. Some experiments in this direction have shown that on incubation of UDP acetylglucosamine with a crude liver extract part of the acetylglucosamine is converted into acetylgalactosamine. We hope to carry out more work on this point.

EXPERIMENTAL

Methods—The methods were in general the same as those described in previous papers from this laboratory (1, 7). For acetylhexosamine a modification of the Morgan-Elson procedure was employed[3] in which potassium borate is used instead of sodium carbonate.

Paper chromatography of sugars was carried out with ethyl acetate-

pyridine-water (6, 20) or butanol-pyridine-water (3:2:1.5) (14). With the latter solvent good separations of galactosamine and glucosamine were obtained, provided the chromatogram was run during at least 38 hours. For the separation of acetylhexosamines the paper was previously immersed in warm 0.2 M sodium tetraborate and air-dried. The chromatograms were sprayed with benzidine-trichloroacetic acid (15) or the modified Elson-Morgan reagent (16).

For the chromatography of nucleotides ethanol-ammonium acetate mixtures (21) were used. The position of ultraviolet-absorbing substances was ascertained with a Mineralight lamp. In all the chromatographic experiments the position of the substances on the paper was referred to the position of an appropriate standard (glucose for free sugars and adenosine for nucleotides), and the results were expressed as the ratio of the distance traveled by the unknown substance to the distance traveled by the standard substance.

Liver Extract—Two bovine livers were obtained within 2 minutes after the death of the animals. In livers obtained 20 minutes or 5 hours after the death of the animals, more nucleosides and fewer nucleotides were found. The livers (8 kilos) were cut in pieces, mixed with dry ice, and taken to the laboratory. They were then cut in smaller portions, and 1 volume of 95 per cent ethanol was added. The mixture was passed through a meat grinder and was then heated with continuous stirring until it boiled. On the following day it was filtered through a 32 cm. Büchner funnel with a filter aid (Hyflo Super-Cel). Prior to chromatography the filtrate was brought to pH 7.5 by the addition of concentrated ammonia.

Ion Exchange Column Chromatography—The alcoholic liver extract (9000 ml., containing about 30,000 μmoles of nucleotides calculated from the absorbancy at 260 mμ as if it were all uridine) was run directly through a Dowex 2-X4 (200 to 400 mesh) column (4.5 × 25 cm.) in the chloride form.

The simplified elution procedure of Cabib *et al.* (7) was used. After washing with water (23 liters), the following solutions were run successively through the column: 0.01 N hydrochloric acid, until the pH of the effluent was 2 (10 liters), 0.02 N sodium chloride in 0.01 N hydrochloric acid (36 liters), until the absorbancy dropped below 0.15, and finally 0.1 N sodium chloride in 0.01 N hydrochloric acid (24 liters). The nucleotides eluted in this fraction amounted to 2000 μmoles calculated as uridine from the absorbancy at 260 mμ. From this solution the nucleotides were adsorbed by stepwise addition of charcoal (Norit A) until the absorbancy became lower than 0.05. The suspension was filtered through a Büchner funnel, and the elution was carried out with 400 ml. of cold 15 per cent pyridine in water. The solution was extracted twice with 200 ml. of chloroform, and the aqueous phase was concentrated to a small volume *in vacuo* at

30–40°. Paper chromatography of this fraction showed that it was composed of UDPG, UDPAG, and several yellow spots, probably flavine nucleotides.

The isolation of the nucleotides was carried out by chromatography on Whatman No. 3 filter paper with ethanol-ammonium acetate of pH 7. The filter paper was previously washed with acetic acid and then with distilled water. After chromatography, the ultraviolet-absorbing zone corresponding to UDPAG from yeast was cut off and eluted with distilled water. The yield was about 150 μmoles.

Identification of Acetylhexosamines—For the quantitative test liver UDPAG was hydrolyzed with 0.01 N hydrochloric acid for 30 minutes at 100°, and acetylhexosamine was determined.

The quantitative estimation of the acetylhexosamine on paper was carried out by Aronson's procedure (19), and the colored substances were extracted at room temperature for 10 minutes with 3 ml. of the following mixture: trichloroacetic acid, 40 per cent (weight per volume) in water, acetic acid, and ethanol (2:2:6).

For the quantitative determination of hexosamines the deacetylation was carried out by heating liver UDPAG in a sealed tube in 2 N hydrochloric acid for 3 hours at 100°. The liquid was then concentrated to dryness *in vacuo*, the residue dissolved in water, and the hexosamine content determined according to Blix (17).

In order to obtain a solution of the sugar suitable for chromatography, UDPAG from liver was hydrolyzed as above, and the liquid was treated successively with a cation exchange and anion exchange resin (Dowex 50 and Amberlite IR-4B). The solution was evaporated *in vacuo* and submitted to paper chromatography (Table I).

For chromatography of the deacetylated products, the samples were treated in the same way as for the quantitative determination. The solutions were then evaporated and directly used for paper chromatography (Table II).

The oxidation of hexosamines to pentoses was carried out according to the procedure of Gardell *et al.* (18), adapted to the micro scale as follows: 0.5 to 1 μmole of hexosamine dissolved in 0.2 ml. of water was mixed with an equal volume of a 0.48 per cent ninhydrin solution in 0.1 M citrate buffer of pH 4.7. The mixture was heated 30 minutes at 100° and cooled. The liquid was then treated with ion exchange resins and chromatographed on paper (Table III).

Uridine Diphosphate—UDPAG from liver was hydrolyzed with 0.01 N hydrochloric acid for 15 minutes at 100°. The acid was neutralized with dilute ammonia, and the solution was used for paper chromatography (Table IV).

Uridine Monophosphate—Liver UDPAG was hydrolyzed with 1 N hydro-

chloric acid for 30 minutes at 100°. The liquid was passed through Dowex 50, and the effluent evaporated *in vacuo* and submitted to paper chromatography (Table IV).

SUMMARY

A preparation of nucleotides from bovine liver was fractionated by chromatography on an anion exchange resin. The compounds contained in one of the fractions were separated by paper chromatography, and the ultraviolet-absorbing zone corresponding to uridine diphosphate acetylglucosamine was eluted and studied in more detail. The product obtained gave a typical uridine spectrum, and behaved like UDPG and UDPAG in giving UDP or UMP on acid hydrolysis.

The hexosamine part was identified as a mixture of acetylglucosamine and acetylgalactosamine by paper chromatography before and after deacetylation and by degradation to the corresponding pentoses. The analytical data corresponded to those of a mixture of UDP acetylglucosamine and UDP acetylgalactosamine.

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FURTHER STUDIES ON THE ACTION OF ESTRADIOL IN VITRO*

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Previous experiments with both homogenates and particulate-free enzyme systems obtained from human placentae had shown that estradiol increases the rate of conversion of citrate to α-ketoglutarate (1, 2). The data suggested that estradiol stimulates a diphosphopyridine nucleotide-linked isocitric dehydrogenase found in the fraction not sedimented by a 60 minute centrifugation at 57,000 $\times$ g. Estrone had a stimulatory effect equal to estradiol; in contrast, estriol, progesterone, diethyl stilbestrol, and testosterone were only one one-thousandth as active.

The present paper reports further attempts to elucidate the rôle of estradiol in stimulating placental metabolism.

Materials and Methods

The methods of obtaining, homogenizing, and centrifuging placentae have been described previously (2). The fraction not sedimented by centrifugation at 57,000 $\times$ g for 60 minutes ($S_{57,000}$) was freshly prepared before each experiment from a placenta obtained directly from the delivery room. This fraction was incubated for a period of 1 hour in conventional Warburg vessels at 37° with 100 per cent oxygen as the gas phase and alkali in the center wells. Each vessel contained 1.0 ml. of the $S_{57,000}$ preparation and 2.0 ml. of a mixture containing 30 μmoles of K^+, 10 μmoles of Mg^{++}, 20 μmoles of Cl^-, 0.75 μmole of diphosphopyridine nucleotide (DPN), and 20 μmoles of phosphate buffered at pH 6.9. Substrates were added at a level of 3.0 μmoles per vessel. *dl*-Isocitrate was added at a level of 6.0 μmoles per vessel. The nitrogen content of each $S_{57,000}$ preparation was determined, and the metabolic activities of each preparation are expressed in terms of its nitrogen content. In all experiments, estradiol-17β was added to alternate vessels as an aqueous suspension to give a final concentration of 4×10^{-6} M. For some experiments, the $S_{57,000}$ fraction was placed in a cellophane sac and dialyzed against 100 volumes

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of 0.25 M sucrose overnight at 5° to remove endogenous substrates and cofactors.

Aliquots of the contents of the vessels were analyzed before and after incubation for (*a*) citric acid by Elliott's modification[1] of the method of Natelson, Pincus, and Lugovoy (3), (*b*) lactic acid by the method of Barker and Summerson (4), and (*c*) α-keto acids by a slight modification of the method of Friedemann and Haugen (5).

Results

The experiments reported previously (2) had shown that the enzymes stimulated by estradiol added *in vitro* are located not in the mitochondria or microsomes but in the fraction which is not sedimented by centrifuging at 57,000 × *g* for 60 minutes ($S_{57,000}$). The enzymes which convert citrate to α-ketoglutarate are (1) aconitase, which converts citrate via *cis*-aconitate to isocitrate, (2) isocitric dehydrogenase, which removes 2 hydrogens to yield oxalosuccinate, and (3) oxalosuccinic decarboxylase, which removes CO_2 to give α-ketoglutarate. The increased rate of conversion of citrate to α-ketoglutarate observed when estradiol is added could result from a stimulation of any one of these enzymes or of some combination of them.

Experiments were undertaken in which the $S_{57,000}$ fraction of placenta was incubated with each of the tricarboxylic acids of the citric acid cycle as substrates (Table I). The addition of estradiol to a reaction mixture containing citrate as substrate resulted in increased utilization of citrate and increased production of α-ketoglutarate, confirming previous results. With *cis*-aconitate as substrate, there was production of both citrate and α-ketoglutarate upon incubation. Added estradiol increased the production of α-ketoglutarate by an amount stoichiometrically equal to the decrease in the production of citrate. In experiments with *dl*-isocitrate as substrate, there was a lesser production of citrate and a greater production of α-ketoglutarate than with *cis*-aconitate as substrate. Estradiol added to the incubation mixture again caused a decreased production of citrate and an increased production of α-ketoglutarate. When isocitrate was the substrate, the estradiol-induced increase in α-ketoglutarate production was 3 to 4 times as great as the decrease in citrate production. The experiments in which potassium oxalosuccinate (freshly prepared from the barium salt) was the substrate showed that α-ketoglutarate production from oxalosuccinate by dialyzed $S_{57,000}$ fractions was unaffected by the addition of DPN, of estradiol, or of both together. The figures for oxalosuccinate have been corrected for the amount of oxalosuccinate decarboxylated to α-ketoglutarate by a mixture in which the $S_{57,000}$ fraction was replaced by

[1] Personal communication from K. A. C. Elliott.

distilled water. These experiments eliminate oxalosuccinic decarboxylase as a site of action of estradiol.

These data suggested that the primary effect of estradiol is a stimulation of isocitric dehydrogenase and that the decreased conversion of isocitrate or *cis*-aconitate to citrate in the presence of estradiol is the secondary result of the greater conversion to α-ketoglutarate. Additional experiments were performed to obtain evidence as to whether aconitase was directly affected by estradiol.

In one series of experiments, fluoroacetate was added to placental homogenates or to placental $S_{57,000}$ fractions incubated with citrate as the

TABLE I

Effect of Estradiol on Metabolism of Tricarboxylic Acids by $S_{57,000}$ Preparations of Placenta

The figures are the means ± the standard error, expressed as micromoles per mg. of N per hour.

Substrate	No. of experiments	Δ citrate		Δ α-ketoglutarate	
		Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
Citrate	30	−0.45 ±0.025	−0.95 ±0.040	+0.29 ±0.042	+0.86 ±0.049
cis-Aconitate	18	+1.65 ±0.07	+1.21 ±0.066	+0.36 ±0.04	+0.79 ±0.095
dl-Isocitrate	11	+0.49 ±0.01	+0.37 ±0.054	+0.63 ±0.07	+1.01 ±0.14
Oxalosuccinate	8			+0.62 ±0.08	+0.62 ±0.16

substrate. Fluoroacetate had no inhibitory effect on either preparation; apparently the placenta does not have the enzymes to convert fluoroacetate to fluorocitrate. The actual inhibitor of aconitase was shown by Peters *et al.* (6) to be fluorocitrate.

Fluorocitric acid was prepared by incubating a mitochondrial preparation of dog kidneys with fluoroacetic acid, malate, and adenosine triphosphate, by a modification of the procedure of Buffa, Peters, and Wakelin (7). The fluorocitrate was isolated from the trichloroacetic acid filtrate of the incubation mixture by precipitation with barium and purification by the method of Peters (8).

When fluorocitrate was added to a dialyzed $S_{57,000}$ preparation of the placenta incubated with citrate as the substrate, the citric aconitase was inhibited. Instead of a disappearance of citrate with time, as in the control, there was an accumulation of citrate (Table II). The production of α-keto acid was decreased by fluorocitrate, and there was no stimulation of

keto acid production by estradiol in the presence of fluorocitrate. There was a greater accumulation of citrate when a higher level of fluorocitrate was added.

In other experiments, fluorocitrate was added to dialyzed placental $S_{57,000}$ preparations incubated with *cis*-aconitate or isocitrate as the substrate. At the same level of inhibitor which decreased α-keto acid production from citrate, the production of α-keto acid from *cis*-aconitate or isocitrate was unimpaired. The addition of estradiol occasioned the usual

TABLE II

Effect of Fluorocitrate on Dialyzed $S_{57,000}$ Preparations of Placenta

Micromoles per mg. of N per hour; means ± the standard error of four experiments.

Substrate	Δ citrate		Δ α-ketoglutarate	
	Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
Citrate, control	−0.32 ±0.03	−0.86 ±0.04	+0.26 ±0.03	+0.79 ±0.02
+ 0.1 ml. fluorocitrate	+1.25 ±0.02	+1.35 ±0.02	+0.16 ±0.01	+0.16 ±0.01
+ 0.5 " "	+5.16 ±0.02	+5.12 ±0.02	+0.26 ±0.03	+0.26 ±0.03
cis-Aconitate, control	+2.40 ±0.05	+2.32 ±0.04	+0.25 ±0.04	+0.78 ±0.08
+ 0.1 ml. fluorocitrate	+1.71 ±0.03	+1.54 ±0.02	+0.46 ±0.05	+1.06 ±0.11
dl-Isocitrate, control	+0.78 ±0.01	+0.62 ±0.02	+0.22 ±0.08	+0.54 ±0.02
+ 0.1 ml. fluorocitrate	+0.36 ±0.04	+0.35 ±0.04	+0.48 ±0.035	+0.82 ±0.078

marked increase in keto acid production (Table II). These experiments provide further proof that aconitase is not the site of estradiol stimulation.

trans-Aconitate, a competitive inhibitor of aconitase (9), was used in a second series of experiments. *trans*-Aconitic acid was purchased from the Nutritional Biochemicals Corporation and recrystallized several times from water, then dried in a desiccator over calcium chloride. A sample of the final product was shown by silica gel chromatography to be free of other organic acids and to be 86 per cent pure *trans*-aconitic acid. When a dialyzed $S_{57,000}$ preparation was incubated with *trans*-aconitate alone, no α-ketoglutarate and no citrate was formed. This placenta fraction thus has no enzyme for the trans-cis isomerization of aconitic acid.

When the $S_{57,000}$ fraction was incubated with citrate as the substrate

(Table III), the addition of *trans*-aconitate (3 μmoles per ml.) greatly decreased both citrate utilization and keto acid production. There was no effect of estradiol on the system containing *trans*-aconitate at this level.

trans-Aconitate added at levels of 0.3, 0.9, 1.0, or 3.0 μmoles per ml. to a reaction mixture containing *cis*-aconitate as substrate did not decrease the conversion of *cis*-aconitate to citrate. However, at 10 μmoles per ml., *trans*-aconitate decreased the rate of this step by 40 per cent. At all levels of *trans*-aconitate tested, the steps from *cis*-aconitate to α-ketoglutarate

TABLE III

Effect of trans-Aconitate on $S_{57,000}$ Preparations of Placenta

Micromoles per mg. of N per hour; means ± the standard error of six experiments. *trans*-Aconitate was added to give a concentration of 3 μmoles per ml. in the incubation medium.

Substrate	Δ citrate		Δ α-ketoglutarate	
	Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
Citrate, control	−0.33	−0.74	+0.26	+0.75
	±0.03	±0.04	±0.04	±0.04
+ *trans*-aconitate	0	−0.09	+0.12	+0.12
		±0.05	±0.01	±0.01
cis-Aconitate, control	+1.83	+1.48	+0.20	+0.93
	±0.11	±0.14	±0.02	±0.10
+ *trans*-aconitate	+1.70	+1.58	+0.38	+0.74
	±0.09	±0.12	±0.05	±0.11
dl-Isocitrate, control	+0.45	+0.37	+0.37	+0.51
	±0.01	±0.02	±0.02	±0.02
+ *trans*-aconitate	+0.43	+0.36	+0.41	+0.58
	±0.02	±0.02	±0.01	±0.02

proceeded at a rate equal to or greater than that of the control. The stimulatory effect of estradiol on the production of α-ketoglutarate was evident at all levels of *trans*-aconitate.

When isocitrate at concentrations of 1 or 10 μmoles per ml. was used as the substrate, a comparable result was obtained. The presence of *trans*-aconitate at a level of 3 μmoles per ml. did not decrease the production of either citrate or α-ketoglutarate. Estradiol increased α-ketoglutarate production from isocitrate in the presence of *trans*-aconitate.

Further evidence that estradiol stimulates isocitric dehydrogenase rather than aconitase comes from experiments in which *cis*-aconitate at levels of 5 μmoles per ml. and 1 μmole per ml. was present as substrate (Table IV). At the higher level, no effect of estradiol on the production of citrate from

cis-aconitate was apparent; yet the effect on the production of α-ketoglutarate from *cis*-aconitate was undiminished. It would appear that the decrease in citrate production which results when estradiol is added to an incubation mixture containing the lower concentration of *cis*-aconitate is the indirect result of increased utilization of *cis*-aconitate in the production of α-ketoglutarate. When an excess of substrate is present, enough to saturate the citric aconitase system, the increased conversion of *cis*-aconitate to α-ketoglutarate induced by the addition of estradiol does not decrease the conversion of *cis*-aconitate to citrate.

The placental isocitric dehydrogenase, like many other dehydrogenases (10), is inhibited by mercurial compounds and presumably has an active sulfhydryl group. When *p*-chloromercuriphenylsulfonic acid was added

TABLE IV

Effect of Substrate Concentration on cis-Aconitate Metabolism by $S_{57,000}$ Preparations of Placenta

Micromoles per mg. of N per hour; means ± the standard error of four experiments.

cis-Aconitate	Citrate produced		α-Ketoglutarate produced	
	Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
μmoles per ml.				
1	+2.48	+2.29	+0.28	+0.62
	±0.03	±0.01	±0.02	±0.04
5	+6.32	+6.32	+0.45	+0.82
	±0.08	±0.10	±0.05	±0.05

at a level of 1 or 2×10^{-4} M, α-ketoglutarate production from isocitrate was decreased and the estradiol effect was minimal (Table V). At 6×10^{-4} or 1×10^{-3} M, *p*-chloromercuriphenylsulfonic acid inhibited α-ketoglutarate production completely. At the lower levels of the mercurial compound, citrate production from isocitrate was increased, presumably as the result of decreased α-ketoglutarate production. At 2×10^{-4} M *p*-chloromercuriphenylsulfonic acid the estradiol effect on citrate production was diminished or absent. These facts are consonant with the hypothesis that the prime action of estradiol is a stimulation of isocitric dehydrogenase. At the higher levels of the mercurial, aconitase is also inhibited, for citrate production is decreased.

Experiments with dialyzed $S_{57,000}$ fractions showed that reduced DPN is unable to replace oxidized DPN in the system. Both DPN and DPNH (dihydrodiphosphopyridine nucleotide, Sigma) were added at levels of 0.75 μmole per vessel. The results of experiments with citrate as substrate are

given in Table VI. When DPN was present, the usual marked effects of estradiol on citrate utilization and α-ketoglutarate production were evident. Both citrate utilization and α-ketoglutarate production were much

TABLE V

Effect of p-Chloromercuriphenylsulfonic Acid on Isocitrate Metabolism by $S_{37,000}$ Preparations of Placenta

Micromoles per mg. of N per hour; means ± the standard error of four experiments.

p-Chloromercuriphenylsulfonic acid in incubation medium	Citrate produced		α-Ketoglutarate produced	
	Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
M				
0	+0.86	+0.67	+0.20	+0.45
	±0.02	±0.02	±0.09	±0.06
1×10^{-4}	+0.91	+0.77	+0.18	+0.25
	±0.02	±0.01	±0.07	±0.05
2×10^{-4}	+1.00	+0.94	+0.12	+0.15
	±0.02	±0.02	±0.04	±0.02
6×10^{-4}	+0.27	+0.29	0	0
	±0.04	±0.03		
1×10^{-3}	+0.22	+0.17	0	0
	±0.01	±0.01		

TABLE VI

Comparsion of Oxidized and Reduced DPN As Cofactor for Isocitric Dehydrogenase

Micromoles per mg. of N per hour; means ± the standard error of four experiments.

Cofactor added, 0.75 μmole per vessel	Citrate utilized		α-Ketoglutarate produced	
	Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
DPN	−0.44	−0.82	+0.33	+0.91
	±0.027	±0.07	±0.01	±0.05
DPNH	−0.19	−0.27	+0.15	+0.12
	±0.05	±0.06	±0.02	±0.01

less with DPNH than with DPN. There was no estradiol effect on α-ketoglutarate production and only a slight one on citrate utilization under these circumstances.

These experiments indicated that a hydrogen acceptor must be present for the estradiol effect to be evident. The system is specific, for triphos-

phopyridine nucleotide cannot be substituted for DPN (2). Further experiments with dialyzed $S_{57,000}$ preparations showed that methylene blue can, to a slight extent, replace DPN in the system. Methylene blue, 0.26 μmole per vessel, added to an anaerobic incubation medium containing dialyzed $S_{57,000}$, DPN, and citrate as substrate, increased citrate utilization and α-ketoglutarate production (Table VII). When estradiol was added to this system, the usual stimulation of citrate utilization and α-ketoglutarate production was observed. When methylene blue without DPN was present, citrate was utilized and α-ketoglutarate was produced,

TABLE VII

Effect of DPN and Methylene Blue on Citrate Metabolism by Dialyzed $S_{57,000}$ Preparation Incubated Anaerobically

Micromoles per mg. of N per hour; means ± the standard error of four experiments.

Substance added	Citrate utilized		α-Ketoglutarate produced	
	Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
Nothing	0	0	0	0
DPN, 0.75 μmole per vessel	−0.12 ±0.02	−0.55 ±0.01	+0.34 ±0.01	+0.76 ±0.06
Methylene blue, 0.26 μmole per vessel	−0.19 ±0.07	−0.27 ±0.03	+0.56 ±0.09	+0.56 ±0.06
DPN + methylene blue	−0.32 ±0.03	−0.87 ±0.02	+0.87 ±0.03	+1.60 ±0.06

but added estradiol had no effect in increasing citrate utilization and α-ketoglutarate production.

Cysteine has been shown to protect aconitase (11) and to reactivate glyceraldehyde-3-phosphate dehydrogenase after inhibition by *p*-chloromercuribenzoate (12). Cysteine at a level of 1 μmole per ml. was added to $S_{57,000}$ preparations containing *cis*-aconitate as substrate, and the production of citrate and of α-ketoglutarate was measured (Table VIII). Cysteine had an effect comparable to DPN; *i.e.*, it increased α-ketoglutarate production and decreased citrate production. DPN and cysteine together had an effect greater than either one alone. The addition of estradiol to these incubation mixtures accelerated α-ketoglutarate production and decreased citrate production only when DPN was present. Estradiol was without effect when cysteine alone was present. However, cysteine did not interfere with the estradiol effect, for estradiol accelerated α-ketoglutarate production when both cysteine and DPN were present. Similar

results were obtained with citrate or with isocitrate as substrate: cysteine increased keto acid production, but estradiol did not produce a further increase in keto acid production in the absence of DPN. The substitution of cystine for cysteine yielded comparable results (Table IX). The cysteine and cystine were obtained from the Nutritional Biochemicals Corporation.

When an $S_{57,000}$ preparation was incubated with cysteine in the absence of substrate, no keto acid was produced. The α-keto acid produced when

TABLE VIII

Effect of Cysteine and DPN on Utilization of cis-Aconitate by $S_{57,000}$ Preparations of Placenta

Micromoles per mg. of N per hour; means ± the standard error of four experiments.

Substance added	Citrate produced		α-Ketoglutarate produced	
	Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
Nothing	+1.70 ±0.04	+1.48 ±0.06	0	0
Cysteine, 3 μmoles per vessel	+1.43 ±0.08	+1.42 ±0.06	+0.34 ±0.04	+0.32 ±0.04
DPN, 0.75 μmole per vessel	+1.42 ±0.08	+1.08 ±0.06	+0.21 ±0.04	+0.55 ±0.08
DPN + cysteine	+1.25 ±0.01	+1.07 ±0.07	+0.47 ±0.07	+0.71 ±0.11

cysteine was added to an incubation mixture containing citrate as substrate was shown to be α-ketoglutaric acid by silica gel chromatography according to the procedure of Bulen, Varner, and Burrell (13).

The exact rôle of cysteine (or cystine) in this system is not clear. It is unlikely that it is acting simply as a protector of aconitase, for it accelerates α-ketoglutarate production from isocitrate as well as from citrate or *cis*-aconitate. It is possible that both cystine and methylene blue act by accepting hydrogens from DPNH, thus making DPN available for further reduction by the isocitric dehydrogenase. If the amount of DPN were rate-limiting, as it presumably is when no DPN has been added to the $S_{57,000}$ preparation, then the rôle postulated for cystine would account for the observed acceleration of α-ketoglutarate production. It would also account for the observation that in the presence of cyst(e)ine estradiol stimulation is evident in the presence, but not in the absence, of DPN.

To test this hypothesis a dialyzed $S_{57,000}$ preparation was incubated anaerobically with *cis*-aconitate as substrate and with added cysteine or

cystine (1 μmole per ml.). No keto acid was produced with or without estradiol in the presence of cysteine. With cystine in the incubation medium, keto acid production was 0.05 μmole per mg. of N per hour in the absence and 0.15 in the presence of estradiol. When DPN was added along with cysteine, keto acid production was 0.16 μmole per mg. of N per hour in the absence and 0.86 in the presence of estradiol. These experiments indicate that cystine, not cysteine, is the active substance, and that it functions as a hydrogen acceptor in the system. The effect of cystine in the absence of added DPN may be mediated by residual, bound DPN in the dialyzed $S_{57,000}$ fraction. Alternatively, cystine might be able to accept hydrogens directly from isocitrate. The apparent effect of

TABLE IX

Effect of Cysteine and Cystine on Utilization of Citrate by $S_{57,000}$ Preparations of Placenta

Micromoles per mg. of N per hour; means ± the standard error of four experiments.

Substance added	Citrate utilized		α-Ketoglutarate produced	
	Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
DPN, 0.75 μmole per vessel	−0.24 ±0.05	−0.77 ±0.11	+0.22 ±0.03	+0.59 ±0.05
Cysteine, 3 μmoles per vessel	−0.24 ±0.10	−0.29 ±0.06	+0.39 ±0.05	+0.31 ±0.03
Cystine, 3 μmoles per vessel	−0.24 ±0.03	−0.33 ±0.04	+0.34 ±0.04	+0.40 ±0.08

cysteine aerobically is presumably due to its spontaneous oxidation to cystine.

DISCUSSION

The present experiments have shown that estradiol accelerates the production of α-ketoglutarate from isocitrate and *cis*-aconitate as well as from citrate. It does not, however, accelerate the conversion of oxalosuccinate to α-ketoglutarate. These facts suggest that estradiol is effective at the isocitric dehydrogenase step and not at steps mediated by aconitase or oxalosuccinic decarboxylase. The decreased production of citrate from *cis*-aconitate or isocitrate observed in the presence of estradiol is believed to result secondarily from the greater utilization of *cis*-aconitate or isocitrate in α-ketoglutarate production.

The experiments with fluorocitrate and *trans*-aconitate provide corroborative evidence that citric aconitase is not stimulated by estradiol. Either fluorocitrate or *trans*-aconitate will inhibit citrate utilization and prevent

the estradiol-induced stimulation of citrate utilization and α-ketoglutarate production with citrate as the substrate. These substances do not, however, decrease the production of α-ketoglutarate from *cis*-aconitate or isocitrate nor do they alter the acceleration of this system induced by added estradiol.

The observation that the system requires a specific hydrogen acceptor, DPN, for estradiol to accelerate α-ketoglutarate production also indicates that estradiol stimulates isocitric dehydrogenase, which is the only dehydrogenation step between citrate and α-ketoglutarate. Corroborative evidence is provided by the experiments which showed that *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonic acid prevent α-ketoglutarate production from isocitrate and also prevent the usual estradiol stimulation of this system. Dehydrogenases are generally inhibited by sulfhydryl inhibitors, and it is reasonable to conclude that these mercurial compounds are inhibiting isocitric dehydrogenase.

These studies have established that reduced DPN cannot substitute for oxidized DPN in this system. This makes it clear that DPN is acting as a hydrogen acceptor. Spectrophotometric determinations made concurrently (14) supply evidence that estradiol does not accelerate the reoxidation of DPNH. The strict requirement of the estradiol-stimulated system for DPN was further shown by experiments in which cystine or methylene blue was used in place of DPN. The increased α-ketoglutarate production from isocitrate observed when cystine or methylene blue was added to a system containing DPN presumably resulted from the transfer of hydrogen from DPNH to these acceptors, thereby making more oxidized DPN available.

The possibility remains that estradiol is involved as a mediator in transferring hydrogens from isocitrate to DPN and that in this process it is rapidly and reversibly converted from estrone to estradiol. There is no direct evidence that this occurs, and the alternative, that estradiol activates isocitric dehydrogenase itself, must also be considered.

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SUMMARY

1. Experiments in which particulate-free enzyme preparations of human placenta are employed provide evidence that estradiol stimulates a DPN-

linked isocitric dehydrogenase. Estradiol increases α-ketoglutarate production from citrate, *cis*-aconitate, or isocitrate, but not from oxalosuccinate. Estradiol decreases citrate production from *cis*-aconitate or isocitrate; presumably this is a secondary result of the increased conversion of the substrate to α-ketoglutarate.

2. Experiments with fluorocitrate or *trans*-aconitate provide further evidence ruling out aconitase as the site of action of estradiol. These substances inhibit aconitase, but do not eliminate the estradiol-induced acceleration of α-ketoglutarate production from *cis*-aconitate or isocitrate.

3. When isocitric dehydrogenase is inhibited by *p*-chloromercuriphenylsulfonic acid, α-ketoglutarate production ceases and estradiol is without effect.

4. Reduced DPN is unable to replace DPN in this system, which indicates that DPN functions as a specific hydrogen acceptor. The results of experiments with cystine and methylene blue provide confirmatory evidence for this hypothesis.

5. The possible mechanism of estradiol stimulation is discussed.

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SPECTROPHOTOMETRIC STUDIES OF THE STIMULATION OF HUMAN PLACENTAL PREPARATIONS BY ESTRADIOL*

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Earlier studies from this laboratory (1, 2) have provided evidence that the metabolism of human placenta is stimulated *in vitro* by the addition of estradiol-17β. A soluble cytoplasmic fraction free of cell particles, obtained by high speed centrifugation of placental homogenates, can utilize substrates such as citrate, *cis*-aconitate, and isocitrate. The addition of estradiol to the system results in an increase in the utilization of the substrate and an increase in the accumulation of α-keto acid over that of the control. Citrate, *cis*-aconitate, and isocitrate are rapidly interconverted by aconitase; the equilibrium constants are such that the formation of citrate is greatly favored (3). When *cis*-aconitate or isocitrate serves as substrate in this system, the production of citrate is less in the vessel containing estradiol than in the control. This was shown to be a secondary result of the increased conversion of these substrates to α-ketoglutarate induced by the added estradiol (4). Diphosphopyridine nucleotide (DPN) was found to be a necessary cofactor for the stimulation of this system by estradiol; triphosphopyridine nucleotide (TPN) was ineffective (2). This suggested that a DPN-dependent isocitric dehydrogenase is present in the soluble supernatant fraction of placenta and that the reaction catalyzed by this enzyme is stimulated by estradiol.

This paper reports the data obtained by observing the oxidation and reduction of DPN by this system spectrophotometrically. By this technique, the pH optimum of the system has been determined, and the effects of added cofactors and metal ions have been investigated. Further experiments demonstrated the presence and influence of coupled enzyme systems in the placental preparation and the reversibility of the DPN-dependent isocitric dehydrogenase reaction in the presence and absence of estradiol.

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Materials and Methods

The reduction of DPN was measured in a Beckman model DU spectrophotometer at a wave-length of 340 mμ. The reaction mixtures were incubated at 22–24° in quartz cuvettes with a 1 cm. light path. A Beckman model H2 pH meter was used for pH measurements. Citric acid analyses were made by a modification[1] of the Natelson, Pincus, and Lugovoy method (5). DPN, 95 per cent pure by assay, was purchased from the Pabst Laboratories; no TPN was present in the preparation. TPN and reduced DPN (DPNH) were purchased from the Sigma Chemical Company. The barium oxalosuccinate was kindly supplied by Dr. Severo Ochoa.

The preparation of the placental cytoplasmic fraction free of mitochondria and microsomes has been described previously (2). This is the fraction not sedimented by a centrifugal force of 57,000 × *g* for 60 minutes ($S_{57,000}$). Because this supernatant fraction undergoes a rapid loss of enzymatic activity upon standing at room temperature, it was kept at 0°. Experiments were carried out as soon as possible after preparation of the enzyme mixture. All constituents of the reaction mixture were brought to room temperature before incubation. In each experiment, the reaction was started by the addition of the pyridine nucleotide.

RESULTS AND DISCUSSION

pH Optimum—The reduction of DPN by the placental enzyme system in the presence and absence of estradiol was measured over a pH range of 6.2 to 8.9. At each pH, three cuvettes were charged with reaction mixtures. The blank cuvette contained 1 ml. of buffer, 6.0 μmoles of *dl*-sodium isocitrate, which had been neutralized with phenol red as indicator, and 1 ml. of the supernatant fraction ($S_{57,000}$) containing approximately 1 mg. of nitrogen. The second cuvette contained buffer, isocitrate, and $S_{57,000}$ fraction plus 0.75 μmole of DPN. The third cuvette contained buffer, isocitrate, DPN, and $S_{57,000}$ preparation plus 3 γ of estradiol, added as an aqueous suspension. The final volume in each cuvette was made up to 3 ml. with distilled water.

Tris(hydroxymethyl)aminomethane (Tris) buffer at a concentration of 0.1 M in the cuvette was used in the pH range 7.0 to 8.9. From pH 6.2 to 7.2 a potassium phosphate buffer with a final concentration of 0.01 M was used. Experiments at pH 7.0 and at 7.2 with either the Tris or the phosphate buffer resulted in identical quantities of DPN reduced.

The optimal pH for DPN reduction in the presence of estradiol was sharply defined at 7.3 (Fig. 1, Curve 1). When estradiol was absent from

[1] Personal communication from K. A. C. Elliott.

the reaction mixture, there was little or no DPN reduction at pH 6.6 to 7.2, but there was an appreciable amount of DPNH produced under more alkaline conditions (Curve 2). Below pH 6.2 there was little or no reduction of DPN in the presence or absence of estradiol. The effect of estradiol on DPN reduction decreased as the pH was increased above 7.3. The increased reduction of DPN by estradiol at a given pH is reflected

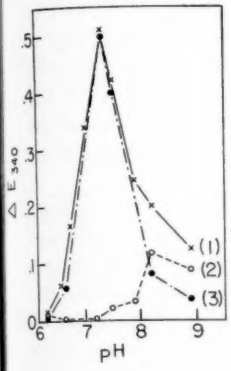

FIG. 1

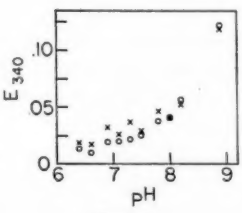

FIG. 2

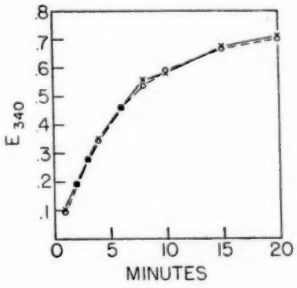

FIG. 3

FIG. 1. Change in optical density of reaction mixtures incubated 60 minutes in presence (×) and absence (○) of estradiol. Wave-length 340 mμ; slit width 1.2 mm.; *d* 1 cm. Each cuvette contained 1 ml. of $S_{57,000}$ preparation, 0.75 μmole of DPN, 6 μmoles of isocitrate, and 1 ml. of buffer; total volume 3 ml. Curve 1 indicates net DPN reduction in the presence of 3 γ of estradiol-17β; Curve 2, net DPN reduction in the absence of estradiol. The increase of DPNH production by estradiol is seen in Curve 3 (●) (Curve 1 minus Curve 2).

FIG. 2. Influence of pH on the optical density of reaction mixtures 2 minutes after the addition of DPN. ×, presence, ○, absence of estradiol.

FIG. 3. Reduction of TPN by placental $S_{57,000}$ preparation in the presence (×) and absence (○) of 3 γ of estradiol. Wave-length 340 mμ; slit width 0.7 mm.; *d* 1 cm. Each cuvette contained 0.2 ml. of $S_{57,000}$ preparation, 0.75 μmole of TPN, 6 μmoles of isocitrate, and 1 ml. of 0.3 M Tris buffer, pH 7.4. Final volume 3 ml.

in the difference in optical density at 340 mμ when the reaction mixture was incubated with and without estradiol (Curve 3). These data show that the estradiol-induced stimulation of DPN reduction is maximal at pH 7.3. Above pH 8, DPN is reduced by other enzymes, *e.g.* lactic dehydrogenase, which are not stimulated by estradiol.

The optical densities at 340 mμ recorded 2 minutes after DPN was added to the reaction mixtures were found to vary directly with the pH of the system in the alkaline range; they were not affected by estradiol (Fig. 2). The absorption at 340 mμ of 0.75 μmole of DPN in 0.1 M Tris buffer was

measured in a series of solutions from pH 7.4 to 9.0. No reduction of DPN was observed. In another experiment, a sample of $S_{57,000}$ preparation was allowed to stand at room temperature for 24 hours to inactivate the enzyme, which responds to estradiol stimulation. Aliquots of this preparation were then incubated with DPN at pH 7.4 and at pH 9.0 in Tris buffer. Optical density readings at 340 mμ after 2 minutes of incubation were comparable to those of Fig. 2 at the respective pH values. No reduction of DPN at pH 7.4 occurred, but the production of DPNH at pH 9.0 was observed. Estradiol did not increase the optical density at either pH. An $S_{57,000}$ fraction was heated to 95° for 30 minutes, the precipitate was centrifuged, and the supernatant fluid was incubated in the usual manner. Again, the optical density at 340 mμ after incubation of the mixture for 2 minutes was increased at the high pH; there was no further reduction of DPN after this initial reading at either pH.

Requirement for DPN—Villee (2) has shown that the soluble supernatant fraction of placental preparations will utilize citrate and accumulate α-ketoglutarate when either DPN or TPN is added to the reaction mixture. Estradiol, however, will increase the utilization of citrate and the accumulation of α-ketoglutarate only when DPN is added; the estradiol stimulation is evident both aerobically and anaerobically. More direct evidence that estradiol does not stimulate the TPN-dependent isocitric dehydrogenase of placenta can be seen in Fig. 3. Added TPN is rapidly reduced in a reaction mixture containing buffer, isocitrate, and 0.2 ml. of $S_{57,000}$ preparation, but the rate of reduction of TPN is not influenced by estradiol.

Effects of Exogenous Substrate—The $S_{57,000}$ preparation presumably contains the intermediates and enzymes of the Embden-Meyerhof cycle as well as other substrates and enzymes. This can be inferred from the data of Fig. 4, for DPN reduction occurred in the absence of added substrate. Indeed, the rate of reduction of DPN was greater in the absence than in the presence of isocitrate. There was, however, an estradiol stimulation of DPNH production with or without added isocitrate. The explanation for greater DPN reduction in the absence of exogenous substrate is not clear.

When similar $S_{57,000}$ fractions were dialyzed for 18 to 24 hours against 0.25 M sucrose at 5° and then incubated, they showed no activity unless a substrate, *e.g.* isocitrate, was added (Fig. 5). The reduction of DPN in the presence of isocitrate without estradiol was greater in the dialyzed than in the non-dialyzed preparation. This, we believe, results from the removal during dialysis of substances such as pyruvate which serve as substrates for the reoxidation of DPNH.

Coupling Systems—Evidence for the presence of a DPN-linked lactic

dehydrogenase in the $S_{57,000}$ fraction of placenta has been presented previously (2). It was thought that this enzyme system could accept hydrogens from reduced DPN if pyruvate were present in the incubation medium. Evidence that this reaction does indeed occur was obtained by observing the rapid reoxidation of reduced DPN upon the addition of 0.1 μmole of pyruvate to the reaction mixture (Fig. 6). The oxidation of

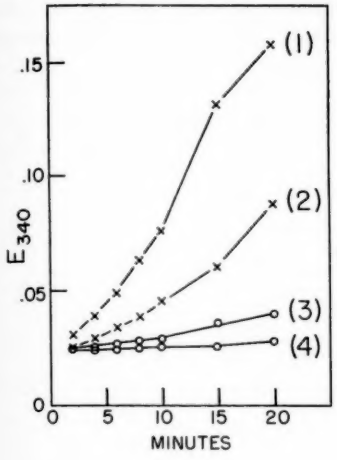

FIG. 4

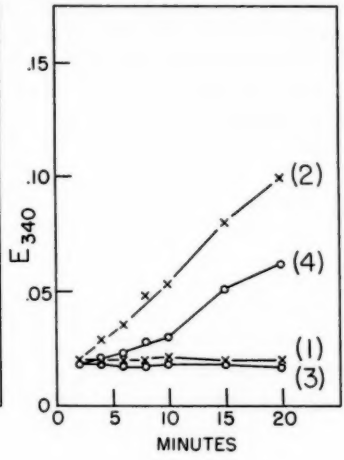

FIG. 5

FIG. 4. Effect of substrate and estradiol on DPN reduction by a non-dialyzed $S_{57,000}$ preparation. Slit width 1.2 mm.; wave-length 340 mμ; *d* 1 cm. Each cuvette contained 1 ml. of 0.3 M Tris buffer, pH 7.4, 1 ml. of $S_{57,000}$ preparation, and 0.75 μmole of DPN. Cuvettes 2 and 4 contained 6 μmoles of isocitrate; Cuvettes 1 and 3 had no added substrate. 3 γ of estradiol were added to Cuvettes 1 and 2. Total volume in each cuvette 3 ml.

FIG. 5. Effect of substrate and estradiol on DPN reduction by a dialyzed $S_{57,000}$ preparation. Conditions and symbols as in Fig. 4.

reduced DPN was completed within 2 minutes after the addition of the pyruvate; thereafter, the reduction of DPN proceeded as before.

This observation led to a search for other DPN-linked dehydrogenases which might reoxidize DPNH in the presence of the proper substrate. The addition of 0.1 μmole of α-ketoglutaric acid to the cuvettes after the incubation had proceeded for 20 minutes resulted in the reoxidation of DPNH (Fig. 7). This reaction, presumably the reduction of α-ketoglutaric to glutamic acid by glutamic dehydrogenase, proceeded at a much slower rate than the lactic dehydrogenase system, for the time required for maximal reoxidation of DPNH was considerably greater. If 0.3 μmole of

α-ketoglutaric acid was added after 20 minutes of incubation, the amount of DPNH oxidized was greater and the time for maximal oxidation of DPNH remained the same, but further DPN reduction did not take place. An excess of α-ketoglutaric acid in the reaction medium inhibited the further oxidation of isocitrate.

The malic dehydrogenase of heart muscle has been shown by Straub (6) to require DPN. Cuvettes containing buffer, $S_{57,000}$ fraction, and DPN were incubated for 20 minutes to produce reduced DPN. The addition of 0.1 μmole of oxalacetic acid at this point resulted in the rapid oxidation of the reduced DPN (Fig. 8).

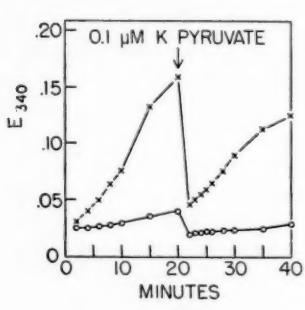

FIG. 6

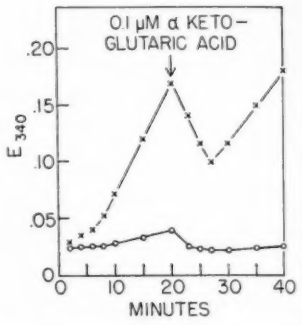

FIG. 7

FIG. 6. Reoxidation of DPNH by potassium pyruvate added 20 minutes after DPN. Wave-length 340 mμ; slit width 1.2 mm.; *d* 1 cm. ×, presence, ○, absence of 3 γ of estradiol. Each cuvette contained 1 ml. of $S_{57,000}$ preparation, 0.75 μmole of DPN, and 1 ml. of 0.3 M Tris buffer, pH 7.4. Total volume of each cuvette 3 ml.

FIG. 7. Reoxidation of DPNH by α-ketoglutaric acid added 20 minutes after DPN. Conditions and symbols as in Fig. 6.

The reoxidation of reduced DPN by the addition of pyruvate, α-ketoglutarate, or oxalacetate could be shown to occur when the incubation medium contained isocitrate as a substrate initially, as well as in the absence of exogenous substrate. This rapid reoxidation of DPNH was also evident when pyruvate was added to a reaction mixture which contained dialyzed $S_{57,000}$.

Effect of Ions—In initial experiments with dialyzed $S_{57,000}$ preparations, it was found that DPN reduction proceeded at a greater rate when a phosphate buffer (pH 6.9) was used than in the presence of Tris buffer (pH 7.3) despite the fact that the optimal pH for DPN reduction in this system is 7.3. When inorganic phosphate was added to the incubation medium containing Tris buffer at pH 7.3, the rate of DPN reduction was

increased. This phosphate effect was of the same magnitude in the presence and absence of estradiol. In 1939, Warburg and Christian (7) found that arsenate could replace inorganic phosphate in the reaction mediated by crystalline yeast 3-phosphoglyceraldehyde dehydrogenase. Sodium arsenate at a final concentration of 10 mmoles per liter was added to reaction mixtures containing Tris buffer to determine whether an analogous system existed in the placental supernatant fraction. Arsenate was found to increase the rate of DPN reduction in our system over that of inorganic phosphate, but the presence of arsenate did not enhance the estradiol effect. These experiments indicate that some DPN-dependent enzyme other than the estradiol-stimulated system of the $S_{57,000}$ fraction requires the phosphate ion.

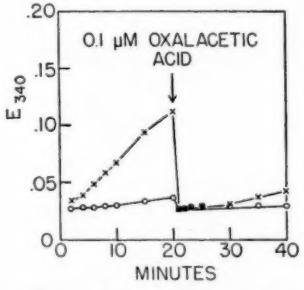

FIG. 8. Reoxidation of DPNH by oxalacetic acid added 20 minutes after DPN. Conditions and symbols as in Fig. 6.

A study of the effect of a number of cations on the reduction of DPN by placental $S_{57,000}$ fraction was undertaken (Table I). None of the cations studied accelerated the rate of DPN reduction unless estradiol was present in the reaction mixture. Calcium almost completely suppressed DPN reduction. In the presence of estradiol, Na^+ and K^+ did not have any effect, but Mn^{++}, Mg^{++}, and Co^{++} caused marked stimulation in the rate of production of DPNH.

Adler *et al.* (8) found that a TPN-dependent isocitric dehydrogenase requires either Mg^{++} or Mn^{++}; the latter is much more effective. With the use of dialyzed extracts of washed, acetone-dried pig heart, Ochoa (9) demonstrated that two reversible enzyme systems are involved. The reaction catalyzed by isocitric dehydrogenase does not require added cation, but the one catalyzed by oxalosuccinic decarboxylase is dependent on the presence of Mg^{++} or Mn^{++}. The activation of DPN-dependent isocitric dehydrogenases from animal tissues (10) and yeast (11) by Mg^{++} and Mn^{++} has been reported. In our system the stimulatory effects of Mn^{++}, Co^{++},

and Mg^{++} were noted only when estradiol was present in the reaction mixture. Since the evidence points to the stimulation of the isocitric de-

TABLE I

Effect of Cations on DPN Reduction by Dialyzed $S_{57,000}$ Fraction in Presence and Absence of Estradiol

Each value is the mean of determinations with three different placental preparations. The incubation period was 30 minutes. Each cuvette contained 1 ml. of 0.3 M Tris buffer, pH 7.4, 1 ml. of dialyzed $S_{57,000}$ preparation, 6.0 μmoles of isocitrate, 0.75 μmole of DPN, and 10 μmoles of the cation, added as the chloride. Total volume in each cuvette 3 ml.

Ion	ΔE_{340}	
	Estradiol absent	Estradiol present
None	0.053	0.073
Na^+	0.026	0.073
K^+	0.020	0.068
Ca^{++}	0.003	0.010
Mn^{++}	0.023	0.262
Mg^{++}	0.057	0.313
Co^{++}	0.063	0.366

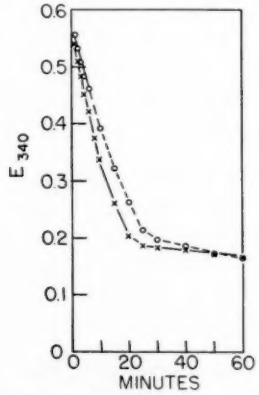

FIG. 9. Oxidation of DPNH by dialyzed $S_{57,000}$ in the presence of oxalosuccinate and in the presence (×) and absence (○) of 3 γ of estradiol. Wave-length 340 mμ; slit width 1.2 mm.; *d* 1 cm. Each cuvette contained 1 ml. of dialyzed $S_{57,000}$ preparation, 0.38 μmole of DPNH, 6.4 μmoles of oxalosuccinate, and 1 ml. of 0.3 M Tris buffer, pH 7.4. Total volume in each cuvette 3 ml.

hydrogenase reaction by estradiol, it is assumed that the action of these cations is associated with isocitric dehydrogenase rather than with oxalosuccinic decarboxylase.

Oxidation of DPNH—Evidence presented in this paper and previous publications (2, 4) strongly suggests that estradiol stimulates the isocitric dehydrogenase system. To investigate the reversibility of this system, dialyzed placental $S_{57,000}$ fraction was incubated with reduced DPN and oxalosuccinate as the substrate. The oxidation of DPNH was measured spectrophotometrically at 340 mμ. Citric acid determinations on aliquots of the reaction mixtures indicated that oxalosuccinate had been converted to citric acid (Table I). This evidence indicates that the isocitric dehydrogenase in the $S_{57,000}$ fraction catalyzes the reversible reaction,

$$\text{Isocitrate} + \text{DPN}^+ \rightleftarrows \text{oxalosuccinate} + \text{DPNH} + \text{H}^+$$

Evidence that estradiol accelerates the reaction to the left as well as to the right is presented in Fig. 9. The oxidation of DPNH occurs more rapidly when estradiol is present. When oxalosuccinate is omitted from the system, DPNH oxidation occurs at a much slower rate and is not influenced by estradiol. The experiments provide further evidence that the reaction catalyzed by isocitric dehydrogenase and not that mediated by oxalosuccinic decarboxylase is stimulated by estradiol. The addition of oxalosuccinate to the reaction mixture after the incubation had proceeded to the stage where DPNH was no longer being oxidized occasioned a sharp increase in DPNH oxidation. This indicated that the concentration of oxalosuccinate in the incubation medium was the limiting factor for DPNH oxidation. At the end of the 1 hour incubation period, DPN (Table II, fourth column) and citric acid (third column) were formed in approxi-

TABLE II

Oxidation of Reduced DPN by Dialyzed $S_{57,000}$ Preparation with Oxalosuccinate As Substrate in Presence and Absence of Estradiol

Each series of reaction cuvettes was set up exactly as described in Table I, except that DPNH replaced DPN and oxalosuccinate replaced isocitrate. The incubation period was 1 hour.

The amounts are in micromoles per vessel.

Experiment No.	DPNH added	Oxalosuccinate added*	Citric acid produced		DPNH oxidized†	
			Estradiol absent	Estradiol present	Estradiol absent	Estradiol present
1	0.75	4.8	0.146	0.105	0.273	0.237
2	0.75	6.4	0.124	0.104	0.255	0.207
3	0.38	6.4	0.099	0.084	0.263	0.265
4	0.38	6.4	0.062	0.056	0.186	0.180

* Corrected for 30 per cent impurities (12).

† Calculated from the molecular extinction coefficient 6.22×10^6 sq. cm. $\times$ mole^{-1} (13).

mately equal amounts in the presence and absence of estradiol. As the incubation continued, the rapid spontaneous decarboxylation of oxalosuccinate (12), the availability of DPN, and the presence of formed isocitrate shifted the reaction to the right.

SUMMARY

The characteristics of the stimulation by estradiol of a human, term, placental fraction which is not sedimented by 57,000 × *g* have been studied spectrophotometrically and chemically.

1. The optimal pH at which the placental preparation is stimulated by estradiol to reduce DPN is 7.3.

2. TPN is rapidly reduced by enzymes in this placental fraction, but the production of TPNH is not influenced by estradiol.

3. At least three different coupling systems are present in the placental preparations which are capable of reoxidizing reduced DPN.

4. Mn^{++}, Mg^{++}, and Co^{++} markedly accelerate the production of DPNH by the estradiol-stimulated enzyme system.

5. Evidence is presented which strongly suggests that estradiol stimulates a DPN-dependent isocitric dehydrogenase in both directions.

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THE AMINO ACID REQUIREMENTS OF MAN

XII. THE LEUCINE AND ISOLEUCINE REQUIREMENTS*

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Quantitative studies with five of the eight amino acids which are essential components of the diet of adult man (*cf.* (1)) have been described in earlier papers of this series. Figures representing the minimal daily requirements, as measured by the state of the nitrogen balance, have been proposed *tentatively* for tryptophan (2), phenylalanine (3), lysine (4), and threonine and methionine (5). The present paper is a continuation of these investigations and is concerned with the minimal needs of the organism for leucine and isoleucine.

EXPERIMENTAL

The methods employed in the conduct of the experiments have been discussed in detail elsewhere (6, 2); consequently, they will be considered only briefly at this time. Healthy young men served throughout as the experimental subjects. The nitrogen of the diets was furnished by mixtures of highly purified amino acids, which were consumed in aqueous solution flavored invariably with filtered lemon juice and sometimes with sucrose. Wafers supplied the major part of the energy and all of the inorganic salts. The other components of the rations consisted of extra sucrose and butter fat, as required in individual cases, and vitamins which were administered in the form of a fish liver oil or concentrate and pills carrying the water-soluble factors. The reader is referred to Paper I of this series (6) for the composition of the salt mixture and baking powder used in making the wafers, and for the daily intakes of the vitamins. One-third of the daily allotment of amino acids and other dietary constituents,

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† Some of the experimental data in this paper are taken from a thesis submitted by Charles H. Eades, Jr., in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the University of Illinois. Present address, School of Biological Sciences, University of Tennessee, Memphis, Tennessee.

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excepting the vitamins, was consumed at each meal. The vitamins were taken either once daily or at the morning and evening meals.

In determining the leucine requirement, five subjects were used. Two of these experiments, illustrating the range of values obtained, are detailed below. Subject R. L. F. received initially a diet composed of amino acid Mixture 183 (Table I), Wafers IV (*cf.* (7)), 271.0 gm. of extra sucrose, and 37.4 gm. of extra butter fat. The daily intake of amino acid Mixture 183 furnished 9.77 gm. of nitrogen, of which 0.62 gm. was derived from the

TABLE I

Composition and Daily Intakes of Amino Acid Mixtures

Component	Mixture 183		Mixture 205	
	As used	N content	As used	N content
	gm.	gm.	gm.	gm.
Valine	3.20*	0.38	3.20*	0.38
Leucine	4.91	0.52	2.20	0.23
Isoleucine	6.16*	0.66	2.80*	0.30
Methionine	2.00*	0.19	2.00*	0.19
Threonine	1.60*	0.19	1.60*	0.19
Phenylalanine	2.40*	0.20	2.40*	0.20
Tryptophan	0.30	0.04	0.30	0.04
Lysine monohydrochloride	2.00	0.31	2.00	0.31
Glycine	28.33	5.28	31.40	5.86
Urea	4.29	2.00	4.29	2.00
	55.19	9.77†	52.19	9.70‡

* Racemic acids.

† Of the nitrogen contributed by this mixture, 0.62 gm. was derived from the D forms of valine, isoleucine, and threonine.

‡ Of the nitrogen contributed by this mixture, 0.44 gm. was derived from the D forms of valine, isoleucine, and threonine.

D isomers of valine, isoleucine, and threonine. The total nitrogen content of the food amounted to 10.09 gm., of which 0.32 gm. was of an unknown nature. The latter had its origin largely in the starch of the wafers. Much smaller amounts were contributed by the lemon juice and butter fat, and by the liver concentrate present in the vitamin pills. The daily energy intake of the subject amounted to 55 calories per kilo of body weight.

Subject D. W. W. received initially a diet consisting of amino acid Mixture 205 (Table I), Wafers IV, 179.0 gm. of extra sucrose, and 29.9 gm. of extra butter fat. The daily allotment of Mixture 205 furnished 9.70 gm. of nitrogen, of which 0.44 gm. was derived from the D isomers of valine,

isoleucine, and threonine. The total nitrogen intake of this subject was 10.04 gm. per day, of which 0.34 gm. was of an unknown nature. The daily energy intake amounted to 57 calories per kilo of body weight. In both experiments, the ratio of calories derived from carbohydrates to those furnished by fats was maintained at 2.6. Furthermore, in these tests, as

TABLE II

Leucine Requirement of Man

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
Subject R. L. F.						
5	78.5	10.09	8.51	0.84	+0.74	4.91 gm. L-leucine
5	80.3	10.09	9.82	0.86	−0.59	1.00 " "
6	79.8	10.09	9.10	0.74	+0.25	1.30 " "
5	80.7	10.09	8.87	0.80	+0.42	1.10 " "
7	79.8	10.09	9.30	0.87	−0.08	2.20 " D-leucine
Subject D. W. W.						
7	67.5	10.04	8.87	0.80	+0.37	2.20 gm. L-leucine
6	69.7	10.04	8.87	0.70	+0.47	1.10 " "
12	69.4	10.04	8.91	0.53	+0.60	0.90 " "
8	69.6	10.04	9.20	0.65	+0.19	0.80 " "
6	69.3	10.04	9.15	0.58	+0.31	0.70 " "
7	69.7	10.04	9.18	0.52	+0.34	0.60 " "
9	69.7	10.04	9.44	0.69	−0.09	0.40 " "
6	70.2	10.04	9.01	0.62	+0.41	0.50 " "

* The initial diets of Subjects R. L. F. and D. W. W. contained amino acid Mixtures 183 and 205, respectively.

well as in those involving isoleucine to be described presently, Cellu flour, which has been administered to all subjects for the purpose of providing a residue in the alimentary tract, was incorporated in the wafers instead of being consumed separately as a water suspension.

The results of the two leucine tests are presented in Table II. With Subject R. L. F., the amino acid under investigation was first supplied at the high level of intake (4.91 gm. daily) used in most of the experiments previously reported (*cf.* (6, 2)). As was anticipated, positive nitrogen balance was established promptly and was so maintained for the duration of

the period. During the next three periods, L-leucine was furnished in daily quantities of 1.00, 1.30, and 1.10 gm., respectively, the total nitrogen of the food being kept constant by appropriate adjustments in the glycine content of Mixture 183. The data in Table II show clearly that 1.10 gm. of L-leucine was the smallest amount which was capable of inducing a positive nitrogen balance, and consequently represents the minimal daily requirement of this subject.

During the final period of the experiment upon R. L. F., L-leucine was replaced by the D isomer at a level of 2.20 gm. daily. Despite the fact that this was equivalent to twice the minimal L-leucine requirement, an average daily nitrogen balance of −0.08 gm. ensued. Furthermore, if one excludes the first 2 days of the period, during which nitrogen retention persisted, and calculates the average balance on the basis of the last 5 days only, a value of −0.29 gm. is obtained. In a second experiment with D-leucine, the details of which are omitted, a subject (H. F. K.) having a minimal L-leucine requirement of 0.70 gm. daily received a daily intake of 1.40 gm. of the D isomer. The nitrogen balance became negative on the 2nd day, and from the 2nd to the 5th (final) day of the test had an average daily value of −0.48 gm. In a previous paper dealing with the *qualitative* rôle of leucine (8), attention was called to the fact that even the *exclusion* of this amino acid from the food induces a rather slow and relatively small response. The *daily* balances recorded for one of the subjects in the earlier paper reveal that the output of nitrogen did not exceed the intake until the 3rd day of leucine deprivation. During the subsequent 5 days, the average daily loss amounted to 0.61 gm. Obviously, these comparisons do not enable one to exclude the possibility that some utilization of D-leucine may occur in man. On the other hand, if this is the case, the amount involved is certainly quite small and of little practical significance in the maintenance of nitrogen equilibrium.

In the second quantitative experiment (Table II), Subject D. W. W. received an initial dose of 2.20 gm. of L-leucine. After 7 days, during which the average nitrogen retention amounted to 0.37 gm., the L-leucine intake was reduced to 1.10 gm. daily, the quantity which had been shown to be capable of meeting the minimal needs of Subject R. L. F. Positive nitrogen balance continued. On the assumption that the minimal requirement of D. W. W. might not differ greatly from that of R. L. F., the level of L-leucine was next decreased to 0.90 gm. This also occasioned a strong retention of nitrogen, despite the fact that the period was extended to 12 days to avoid the possibility that a delayed effect might be overlooked. During subsequent periods, the L-leucine dosage was diminished very gradually, as shown in Table II. Contrary to expectations, a negative nitrogen balance did not occur until an intake of 0.40 gm. had been reached. Even

then, the deficiency did not manifest itself in the nitrogen balance until the 3rd day of the period. The average daily loss for the last 7 days amounted to 0.27 gm. During the final period, an increase in the daily dosage to 0.50 gm. resulted in a strongly positive balance. Evidently, the latter quantity represents the minimal requirement of this subject.

As mentioned above, three additional experiments with L-leucine were carried out. The detailed data are omitted to conserve space, but the final results will be presented later in this paper.

In establishing the isoleucine requirement of man, four experiments were conducted, two of which are recorded below. This amino acid is one of the most satisfactory for investigations of this sort, since slight changes in the quantity consumed, when the critical level of intake is approached, induce prompt and distinct alterations in the state of the nitrogen balance. Doubtless this is associated with the fact that the *exclusion* of isoleucine from the food is followed immediately by a negative balance of greater magnitude than that encountered in any other type of amino acid deficiency in man (9). In this respect, isoleucine differs astonishingly from leucine.

Subject W. H. P. served in the first isoleucine test. His initial diet was composed of amino acid Mixture 183 (Table I), Wafers IV, 152.5 gm. of extra sucrose, and 18.1 gm. of extra butter fat. As pointed out above, the daily allotment of amino acid Mixture 183 furnished 9.77 gm. of nitrogen. The total nitrogen content of the diet was 10.08 gm., of which 0.31 gm. was of an unknown nature. The daily energy intake amounted to 55 calories per kilo of body weight, and the ratio of calories derived from carbohydrate to those originating in fat was 2.6.

In the second isoleucine experiment, D. W. W., who had served in one of the leucine tests, again was the subject. As shown in Table II, this young man increased in weight appreciably during the course of the leucine experiment. A further gain occurred during the 6 weeks which intervened between the two investigations in which he participated. However, no change was made in his initial diet; consequently, on a weight basis, his energy intake during the isoleucine experiment was somewhat lower, and amounted to slightly less than 54 calories per kilo.

The results of the two isoleucine experiments are presented in Table III. The requirements were first measured with the racemic amino acid. Later, the effects of the optical isomers were determined. With Subject W. H. P., the data show that during the first period, when the daily intake of DL-isoleucine was 6.16 gm., a strong nitrogen retention occurred. This, of course, was anticipated, since the above quantity had been used routinely in most of the earlier human investigations (3–5). During the second period, a decrease in the intake of isoleucine to 1.20 gm. daily induced a decidedly nega-

tive balance. This effect was reversed promptly during the third period when the dosage was raised to 1.40 gm. daily. The further increase to 1.60 gm. per day during the fourth period was a precautionary measure. In nitrogen balance experiments, one cannot always predict the outcome of a test at a given level, even on the last day of the period, since the feces do not become available for analysis until approximately 24 hours after the urine collection has been completed. In the case under consideration, 1.40

TABLE III

Isoleucine Requirement of Man

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
Subject W. H. P.						
5	67.1	10.08	8.87	0.81	+0.40	6.16 gm. DL-isoleucine
5	67.1	10.08	9.70	0.73	−0.35	1.20 " "
6	67.1	10.08	9.33	0.64	+0.11	1.40 " "
4	68.0	10.08	9.25	0.50	+0.33	1.60 " "
4	68.0	10.08	11.20	0.81	−1.93	2.70 " D-isoleucine
9	67.1	10.08	9.39	0.65	+0.04	0.70 " L-isoleucine
Subject D. W. W.						
4	72.1	10.04	9.17	0.62	+0.25	2.80 gm. DL-isoleucine
5	71.6	10.04	8.75	0.57	+0.72	1.40 " "
10	71.6	10.04	9.50	0.72	−0.18	1.20 " "
5	72.0	10.04	9.10	0.62	+0.32	1.30 " "

* The initial diets of Subjects W. H. P. and D. W. W. contained amino acid Mixtures 183 and 205, respectively.

gm. daily proved to be sufficient; hence, a test at the higher intake could have been omitted but for the fact that this could not be foreseen at the time the dietary alteration was made. Obviously, 1.40 gm. of DL-isoleucine comprised the smallest daily dosage capable of maintaining a positive nitrogen balance.

The results obtained during the last two periods of the experiment upon Subject W. H. P. are particularly interesting and illuminating. As will be observed, replacing DL-isoleucine by 2.70 gm. daily of the D isomer led to a profound deficiency, with an average daily nitrogen loss of 1.93 gm. This

is quite comparable to the effects observed when a subject is *deprived* of isoleucine (9), and proves beyond question that the D isomer is unavailable for the uses of the human organism. Moreover, this conclusion was substantiated by the response of the subject during the last period of the experiment, when he received a daily intake of 0.70 gm. of L-isoleucine. Positive nitrogen balance resulted, as was to be expected on the assumption that only half of the racemic acid is utilized. Attention should be called to the low figure of 0.04 gm. representing the *average* daily retention of nitrogen during the final period. This is really misleading and requires an explanation. As a matter of fact, the deficiency during the penultimate period had been so severe that positive nitrogen balance was not reestablished until the 5th day of L-isoleucine administration. This is precisely the behavior of a subject who has been deprived of isoleucine and then receives the amino acid (*cf.* (9)). A *trend* toward balance always begins immediately after the return of the missing amino acid to the food, but balance is not actually attained for several days. Under these circumstances, the average fails to reveal the true situation. In the experiment upon W. H. P., if one omits the first 4 days of the final period, during which the urinary nitrogen output was diminishing but had not yet reached its normal level, and calculates the balance on the basis of the last 5 days only, a value of +0.25 gm. is obtained.

The effects of D- and L-isoleucine were checked in a second experiment, the details of which are omitted. This subject (L. W. B.) also had a minimal daily requirement of 1.40 gm. of the racemic amino acid. The administration of 2.70 gm. of D-isoleucine again induced an intensely negative balance, which was reversed by the daily consumption of 0.70 gm. of the L isomer. On the basis of the findings in these two experiments, one is warranted in concluding that half of the minimal daily dosage, as determined by the use of DL-isoleucine, represents the minimal L-isoleucine requirement of the individual.

The experiment upon Subject D. W. W. (Table III) was of short duration but yielded an exceedingly clear-cut decision. The tabular data demonstrate that a daily intake of 1.40 gm. of DL-isoleucine induced a strongly positive nitrogen balance, and that 1.20 gm. daily resulted in a moderately negative balance. The fact that the lower intake did not occasion a greater loss of nitrogen suggested the possibility that an intermediate dosage of 1.30 gm. daily might be sufficient. Indeed, the administration of this quantity during the final period enabled the subject to attain a positive balance with an average daily retention of 0.32 gm. of nitrogen. One may conclude, therefore, that the minimal L-isoleucine requirement of this young man was 0.65 gm. daily.

The results obtained in all of the leucine and isoleucine experiments are

summarized in Table IV. As with the amino acids previously considered (2–5), no correlation was detected between the minimal daily requirements of the subjects and their body weights, body surfaces, or creatinine output. However, the *range* of leucine values deserves further comment. The minimal requirements of the subjects for this amino acid varied from 0.50 to 1.10 gm. daily. This is the most pronounced divergence encountered in any of our amino acid investigations, even surpassing slightly that observed in the lysine experiments (4). The data in Table IV show that the high

TABLE IV

Summary of Leucine and Isoleucine Experiments

Subject	Body weight	Minimal daily requirement
	kg.	*gm.*
L-Leucine requirement		
H. F. K.	64.0	0.70
D. W. W.	67.5	0.50
C. H. E.	68.5	0.50
J. R. G.	70.3	0.70
R. L. F.	78.5	1.10
L-Isoleucine requirement		
L. W. B.	58.5	0.70
W. H. P.	67.1	0.70
C. H. E.*	67.6	0.70
D. W. W.*	72.1	0.65

* Subjects C. H. E. and D. W. W. served in both the leucine and isoleucine experiments, but the body weight of each differed at the beginning of the two tests, particularly in the case of D. W. W., who had gained considerably.

figure obtained with Subject R. L. F. was chiefly responsible. This young man had a requirement which exceeded considerably the values observed with the other four subjects. In view of this, it is appropriate to emphasize anew that the findings in each experiment were unequivocal. This is illustrated by the quantitatively dissimilar results obtained in the two experiments recorded in Table II. In each, the response of the subject to alterations in the leucine intake permits of a single interpretation with respect to his minimal requirement. It may be affirmed without reservation, therefore, that the observed differences are real, and did not arise through technical defects or errors in the experimental procedure. On the other hand, one cannot escape the conviction that, had another subject been se-

lected fortuitously in place of R. L. F., the range of leucine requirements shown in Table IV might have fallen within narrower limits. Considerations of this nature stress the provisional character of the figures, and explain why, in the interest of prudence, the *highest* value observed with each amino acid, instead of the mean, has been designated as the minimal requirement. This point of view, we believe, does not minimize the significance of the findings recorded in this and the other papers of this series, all of which are presented with complete confidence. Rather, it is intended to remind the reader that nitrogen balance experiments in man must of necessity be limited to a very small sample of the human race. In consequence thereof, interpretations and conclusions are subject to modification in the light of broader experience acquired with a larger number of individuals.

In contrast to the observations with leucine, the figures representing the isoleucine requirements (Table IV) are the most uniform encountered to date. The possibility cannot be excluded that the minimal needs of other young men for this amino acid might differ somewhat from those herein reported; but the odds are against the likelihood that deviations comparable to those characteristic of leucine and lysine will ever be observed with isoleucine.

In accordance with the practice followed throughout these studies, it is proposed that 1.10 gm. of L-leucine and 0.70 gm. of L-isoleucine be regarded *tentatively* as the minimal daily requirements of adult man for these amino acids. In the formulation of diets designed to exclude amino acid deficiencies, due allowance should be made for the possibility that individuals other than those tested may have still higher needs. For this purpose, twice the tentative minima, or 2.20 gm. of L-leucine and 1.40 gm. of L-isoleucine, are certainly *safe* daily intakes (*cf.* (2)). With racemic amino acids, one must take into account the fact that little, if any, D-leucine and no measurable amount of D-isoleucine are utilized by man. To date, fifteen different individuals have been maintained in positive nitrogen balance on daily intakes of 2.20 gm. or less of L-leucine, and fourteen different individuals have been so maintained on daily intakes of 2.80 gm. or less of DL-isoleucine. In no instance has a requirement in excess of either of the above quantities been encountered.

SUMMARY

Investigations have been conducted upon normal young men for the purpose of determining their minimal leucine and isoleucine requirements as measured by the state of the nitrogen balance. Five subjects served in the leucine and four in the isoleucine experiments. With leucine, the minimal daily requirements were found to be 0.50, 0.50, 0.70, 0.70, and 1.10 gm., respectively, of the L-amino acid. The spread in values proved to be

greater than has been encountered in like experiments with other amino acids, but no correlation could be detected between the minimal needs of the subjects and their body weights, body surfaces, or creatinine output. Tests with D-leucine showed that very little, if any, of this compound is utilized by man. Even when the diet furnished 2.20 gm. daily, positive nitrogen balance could not be attained.

In contrast to the above findings, the tests with isoleucine yielded extraordinarily uniform results. The minimal requirements were 1.40, 1.40, 1.40, and 1.30 gm., respectively, of the *racemic* amino acid. Tests with D- and L-isoleucine showed the former to be totally without influence upon nitrogen balance, and the latter to possess twice the activity of the racemic amino acid. In view of these observations, the minimal daily requirements may be expressed in terms of L-isoleucine as 0.70, 0.70, 0.70, and 0.65 gm., respectively.

Because of considerations discussed in this and preceding papers of this series, the highest observed values, or 1.10 gm. of L-leucine and 0.70 gm. of L-isoleucine, are designated *tentatively* as the minimal daily requirements of man. In formulating diets designed to prevent deficiencies, allowance should be made for the possibility that individuals other than those tested may require somewhat larger intakes. For this purpose, twice the above quantities, or 2.20 gm. of L-leucine and 1.40 gm. of L-isoleucine, are certainly *safe* daily intakes.

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ON THE MECHANISM OF INHIBITION OF XANTHINE OXIDASE BY THE SUBSTRATE XANTHINE*

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In contrast to the behavior of most enzymes, the activity of xanthine oxidase decreases with increasing substrate concentrations even at concentrations of 10^{-4} M or lower (1). Partly on the basis of this exceptional behavior, it has been suggested (2) that xanthine oxidase does not comply with the Michaelis theory of enzyme action. However, inhibition by the substrate, which is shown by many enzymes, is not incompatible with this theory (see Huennekens (3)) and it appears that xanthine oxidase is exceptional only in that this inhibition occurs at unusually low substrate concentrations.

In previous work on this enzyme (4) it was shown that a series of pteridines (*e.g.* isoxanthopterin) inhibits the enzyme by competition with the substrate (xanthine). In the present investigation the inhibition by isoxanthopterin has been used to obtain information regarding the mechanism of inhibition by the substrate.

Theoretical[1]

Mechanism A. Competitive Substrate Inhibition

In other enzyme systems it has been suggested (*e.g.* (5)) that inhibition by the substrate is caused by competition of 2 substrate molecules for a

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[1] The symbols used in the equations have the following denotations: v, v_i, initial reaction rate in the absence and in the presence of the inhibitor; V_m, hypothetical reaction rate if all the enzyme were in the form of the active complex; $v_{max.}$, reaction rate at the optimal substrate concentration; S, I, concentration of the substrate and of the inhibitor. $K_s = E \cdot S/ES$, $K_{ss} = ES \cdot S/ESS$, and $K_i = E \cdot I/EI$, where E, ES, ESS, and EI represent the concentrations of free enzyme and of the respective complexes. The relationships between reaction rate and substrate concentration are derived directly from E_t (concentration of total enzyme) $= E + ES + ESS + EI +$ ...by expressing the right-hand terms as functions of ES (active complex) on the basis of the different equilibria involved and subsequent application of $E_t/ES = V_m/v$. Steady state treatment does not, in all cases, give the same result as equilibrium treatment (6). Many of the resulting rate equations can be found

two-point attachment to the same active center on the enzyme molecule, resulting in the formation of the complexes *ES* (active) and *ESS* (inactive). In this case

$$V_m = v + v \cdot \frac{K_s}{S} + v \cdot \frac{S}{K_{ss}} \tag{1}$$

while[2]

$$v_{max.} = V_m / \left(1 + 2\sqrt{K_s/K_{ss}}\right) \tag{2}$$

If such a system contains an inhibitor which combines with the active center, different possibilities arise.

(*a*) If it forms only the complex *EI*,

$$V_m = v_i + v_i \cdot \frac{K_s}{S} + v_i \cdot \frac{S}{K_{ss}} + v_i \cdot \frac{I \cdot K_s}{K_i \cdot S} \tag{3}$$

and

$$v_{max.} = V_m / \left(1 + 2\sqrt{K_s(1 + I/K_i)/K_{ss}}\right) \tag{4}$$

A linear expression, convenient to work with in experiments with constant substrate and varying inhibitor concentrations, is obtained from Equation 1 and Equation 3.

$$v = (v - v_i) + K_i \cdot (v - v_i)\left(1 + \frac{S}{K_s} + \frac{S^2}{K_s \cdot K_{ss}}\right) \Big/ I \tag{5}$$

A plot of the inhibition $(v - v_i)$ against $(v - v_i)/I$, at constant substrate concentration, yields a straight line with slope $K_i(1 + S/K_s + S^2/K_{ss} \cdot K_s)$. One intercept equals the rate of the reaction (v) without inhibitor. It can be seen that the slope is a non-linear function of S and it is found that

$$\frac{\Delta \text{ slope}}{\Delta S} = K_i\left(1 + \frac{S_1 + S_2}{K_{ss}}\right) \Big/ K_s \tag{6}$$

where S_1 and S_2 represent two different substrate concentrations.

(*b*) If in addition to *EI* the inactive complex *EIS* is formed by combination of *EI* with *S* or otherwise, it is found that a plot of $(v - v_i)$ *versus* $(v - v_i)/I$ still is linear, but again the slope of the plot is a non-linear function of *S*.

(*c*) If the complex *EII* were taken into consideration, it can be shown that the plot would be non-linear.

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in the literature, although most often in a form inverted with respect to v. They are given in the present form because the pertaining plots have finite intercepts with both coordinates and are, therefore, less apt to obscure deviations from linearity (7). $v_{max.}$ is obtained from $dv/dS = 0$ or from $dv_i/dS = 0$.

[2] Haldane and Stern (8) state that $v_{max.} = V_m/(1 + \sqrt{K_s/K_{ss}})$. This must be an error.

Mechanism B. Non-Competitive Substrate Inhibition

Aside from Mechanism A, a non-competitive type of inhibition could be considered. Such a mechanism might be depicted as follows: The substrate combines independently with the active group proper and an auxiliary[3] group (x), resulting in the formation of the complexes ES, ES_x, and ESS_x. In contrast to Mechanism A, not more than 1 substrate molecule is attached to a particular group on the enzyme molecule.

The counterpart of Equation 1 is

$$V_m = v + v \cdot \frac{K_s}{S} + v \cdot \frac{S}{K_{ss}} + v \cdot \frac{K_s}{K_{ss}} \tag{7}$$

or

$$V_m = v\left(1 + \frac{K_s}{S}\right)\left(1 + \frac{S}{K_{ss}}\right)$$

while

$$v_{max.} = V_m/\left(1 + \sqrt{K_s/K_{ss}}\right)^2 \tag{8}$$

When an inhibitor is added, this mechanism offers the following three possibilities.

(a) The inhibitor combines with the active group proper to give the complexes ES, ES_x, ESS_x, EI, and EIS_x, of which only ES is active. The counterpart of Equation 3 can be written as

$$V_m = v_i\left(1 + \frac{K_s}{S}\right)\left(1 + \frac{S}{K_{ss}}\right) + K_s \cdot I \cdot \left(1 + \frac{S}{K_{ss}}\right) \cdot \frac{v_i}{K_i \cdot S} \tag{9}$$

In this case

$$v_{max.} = V_m/\left(1 + \sqrt{K_s(1 + I/K_i)/K_{ss}}\right)^2 \tag{10}$$

By equating Equation 7 and Equation 9 the term $(1 + S/K_{ss})$ cancels and one obtains

$$v = (v - v_i) + K_i\left(1 + \frac{S}{K_s}\right) \cdot \frac{(v - v_i)}{I} \tag{11}$$

As would be expected, this equation is identical with the one derived (9) on the basis of the assumption that no inhibition by the substrate occurs, since inhibition by I is independent of inhibition by S. The slope of a plot of

[3] An "auxiliary" group is designated as one that does not take part in the binding of the substrate to form the active complex ES, but that, nevertheless, is essential for activity to occur. Interaction (electron transport) between S and the auxiliary group, to which the electron acceptor could be attached, might occur through the protein (see "Discussion").

$(v - v_i)$ *versus* $(v - v_i)/I$ at constant substrate concentration equals $K_i(1 + S/K_s)$ while

$$\frac{\Delta \text{ slope}}{\Delta S} = \frac{K_i}{K_s} \tag{12}$$

This ratio is independent of S, in contrast to Mechanism A.

(*b*) The inhibitor combines with the auxiliary group only. The complexes ES, ES_x, ESS_x, EI_x, and ESI_x would obtain, and the following expression can be derived.

$$V_m = v_i\left(1 + \frac{K_s}{S}\right)\left(1 + \frac{S}{K_{ss}}\right) + I\cdot\left(1 + \frac{K_s}{S}\right)\cdot\frac{v_i}{K_i} \tag{13}$$

From this equation it follows that

$$v_{\max.} = V_m/\left(\sqrt{1 + I/K_i} + \sqrt{K_s/K_{ss}}\right)^2 \tag{14}$$

From Equation 7 and Equation 13 one obtains

$$v = (v - v_i) + K_i\left(1 + \frac{S}{K_{ss}}\right)\cdot\frac{(v - v_i)}{I} \tag{15}$$

in the derivation of which, the term $(1 + K_s/S)$ cancels. The slope of the plot now represents $K_i(1 + S/K_{ss})$ while

$$\frac{\Delta \text{ slope}}{\Delta S} = \frac{K_i}{K_{ss}} \tag{16}$$

(*c*) The inhibitor combines with both groups. In addition to the complexes already considered, the complex EII_x is also possible. If its concentration is not negligible with respect to E_t, it can be shown that in this case a plot of $(v - v_i)$ *versus* $(v - v_i)/I$ does not give a straight line.

EXPERIMENTAL

Preparations

Xanthine oxidase was prepared from the whey of raw milk by the following procedure. The whey was filtered over a layer of Celite several inches thick. The filtrate contained no activity, but its dry weight was about 60 per cent of that of the whey. The Celite was washed with an amount of water equal to the original whey. The filtrate then contained less than 10 per cent of the original activity but about 25 per cent of the original dry weight. The enzyme was subsequently eluted from the Celite by 0.1 M pyrophosphate buffer of pH 8.5, resulting in recovery of 60 to 70 per cent of the original activity. The eluate was kept frozen and lost little or none of its activity over a period of more than 6 months. When necessary it was dialyzed. It may be noted that concentrating of the enzyme is not

necessary for activity determination, since the original whey had to be diluted about ten times to obtain a convenient concentration of the enzyme.

Xanthine (substrate) was obtained commercially and purified (4).

Isoxanthopterin (inhibitor) was the same preparation as used previously (4) and was furnished by Dr. George Hitchings of the Wellcome Research Laboratories.

Activity Determinations

The reaction product (uric acid) was measured directly in the reaction mixture by the method of Kalckar (10). The measurements were made in a Beckman spectrophotometer, model DU, at 30° (thermospacers) and pH 8.0 (Veronal buffer). A 10^{-4} M solution of xanthine at this pH yields a $\Delta E_{1\text{ cm.}}^{300\text{ m}\mu}$ of 0.9. The lower limit of substrate concentrations at which initial reaction rates can be determined, therefore, lies between 10^{-4} and 10^{-5} M. The upper limit caused by absorption of the substrate itself is 10^{-3} to 10^{-2} M, but it can be raised by reading at slightly higher wavelengths.

Strict proportionality between observed activity and enzyme concentration is obtained during at least 20 minutes after mixing of enzyme and ubstrate.

Results

No Inhibitor

Curve 1 in Fig. 1, Part I, shows the influence of the substrate concentration on the reaction rate. Whichever mechanism is involved, the shape of the curve (decreasing reaction rates) is mainly determined by K_{ss}. The part of the curve representing increasing reaction rates can barely be reached because initial rates cannot be measured at sufficiently low substrate concentrations. The constants K_s and V_m, therefore, cannot be determined by the usual procedure. However, they can be obtained as follows.

At high substrate concentrations Equation 1, representing Mechanism A, becomes linear with respect to the variables v and $v \cdot S$, since then $S >> K_s$. On this basis, in Fig. 1, Part II, $V_m = 6.5$ and $K_{ss} = 1.0 \times 10^{-3}$. With $v_{max.} = 5.1$ and the aid of Equation 2, K_s is calculated to be 2×10^{-5}.

If the substrate would inhibit by Mechanism B, Equation 7 would simplify to $V_m/(1 + K_s/K_{ss}) = v + v \cdot S/(K_{ss} + K_s)$ when $S >> K_s$. Accordingly, in this case also a plot of v *versus* $v \cdot S$ would then become linear. From the slope and the intercept of the plot in Fig. 1, Part II, and with the aid of Equation 8, values for V_m, K_{ss}, and K_s are found that are identical,

within experimental error, with those estimated on the basis of Mechanism A. This is due to the fact that the ratio, $K_s/K_{ss} = 0.02$, is too small to be of practical significance. Under such circumstances Equation 1 and Equation 7 become identical and the data are consistent with either mechanism.

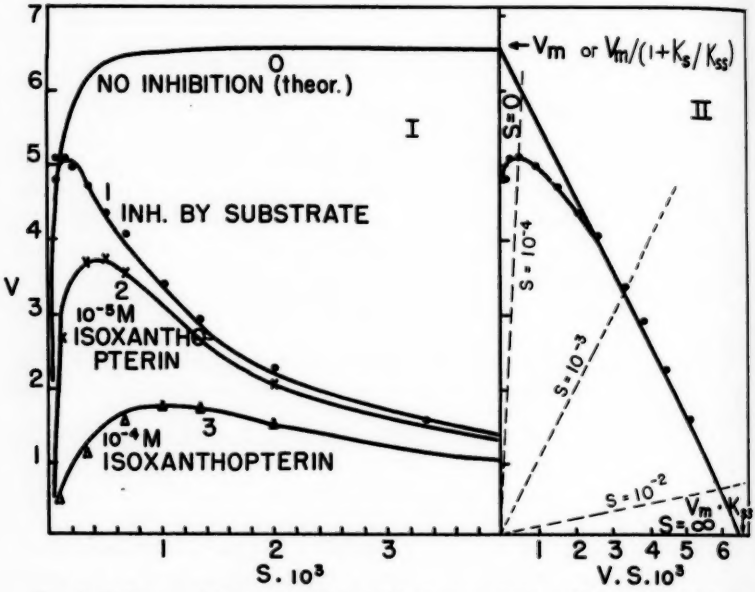

FIG. 1. Influence of the substrate (xanthine) concentration (S) on the initial reaction rate (v, relative values) of xanthine oxidase. All solid curves are theoretical (see the text); the points are experimental. Part I, Curve 0, theoretical curve if no inhibition by the substrate would occur; Curve 1, substrate only; Curves 2 and 3, substrate plus isoxanthopterin. Part II, data of Curve 1 (Part I) plotted as v *versus* $v \cdot S$, showing linearity at high substrate concentrations ($S >> K_s$).

It can be concluded from this section, therefore, that the sole relationship between v and S is inadequate to furnish information regarding the mechanism of substrate inhibition.

Isoxanthopterin As Inhibitor

When an inhibitor is present, the two mechanisms do not necessarily become indistinguishable when K_s/K_{ss} is small. For instance, Equation 3 and Equation 9 differ by the term $v_i \cdot K_s(1 + I/K_i)/K_{ss}$, which cannot be neglected, since I/K_i may be large. It is likely, therefore, that under such conditions more information about the mechanism can be obtained.

For this reason, isoxanthopterin, which inhibits the reaction by competition with the substrate and is not a substrate itself (4), was included in the system.

TABLE I

Values of K_i with Mechanisms Aa, Ba, Bb

I	K_i		
	Mechanism Aa	Mechanism Ba	Mechanism Bb
M			
10^{-5}	1.9×10^{-6}	2.6×10^{-6}	2.6×10^{-5}
10^{-4}	1.1×10^{-6}	2.5×10^{-6}	4.3×10^{-5}

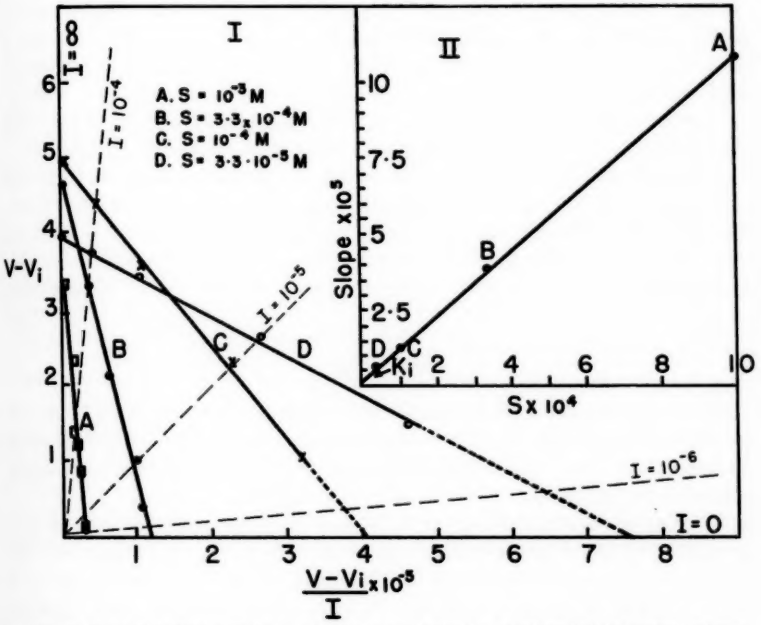

FIG. 2. Part I, inhibition ($v - v_i$) of the activity (v, relative initial rates) of xanthine oxidase by isoxanthopterin in varying concentrations (I). Part II, influence of the substrate concentration (S) on the slope of the plots (Curves A, B, C, and D) of Part I.

Constant Inhibitor and Varying Substrate Concentration—Curves 2 and 3 in Fig. 1, Part I, were obtained with 10^{-5} and 10^{-4} M isoxanthopterin, respectively, included in the system. The data are sufficient only to

obtain evidence for the correctness of the mechanisms (Aa, Ba, Bb) in which only one inhibitor enzyme complex is postulated. From the $v_{max.}$ values (3.7 and 1.7, respectively) and the K_{ss} and K_s ones found above, K_i has been calculated with the aid of Equations 4, 10, and 14 representing these mechanisms. The results are presented in Table I.

Since the same value should be obtained regardless of the inhibitor concentration, Mechanisms Aa and Bb would be excluded. When the complete curves (Fig. 1, Part I) were calculated on the basis of Mechanism Ba, with a K_i of 2.55×10^{-6}, they were found to fit the other experimental data.

Constant Substrate and Varying Inhibitor Concentration—Further and independent evidence for the correctness of Mechanism Ba was found from experiments recorded in Fig. 2. On the basis of Equations 5, 11, and 15 the inhibition $(v - v_i)$ has been plotted against $(v - v_i)/I$ at four different substrate concentrations (Curves A, B, C, D) ranging from 10^{-4} to 3.3×10^{-5} M. In each case almost perfect linearity is obtained over a 100-fold concentration of the inhibitor. This excludes Mechanisms Ac and Bc.

The intercept in each case is equal to v, which is proof that the system contains only one active complex;[4] *i.e.*, the complex EIS is inactive.

Linearity would be obtained for all the other mechanisms but not the constancy of Δ slope/ΔS (see "Theoretical"). As can be seen in the following data, this ratio is constant within 5 per cent of the average, 0.11.

Combination of curves	A and B	A and C	A and D	B and C	B and D	C and D
$\frac{\Delta \text{ slope}}{\Delta S}$	0.107	0.108	0.108	0.114	0.113	0.108

If Mechanism Aa applied, these values would differ by as much as 100 per cent (Equation 6), and no linearity would be obtained with respect to the slope and S as variables (Fig. 2, Part II). Mechanism Ab also can be excluded on this basis.

This leaves Mechanisms Ba and Bb as the only possibilities (see Equations 12 and 16) of the ones that were considered. A choice can be made from the following consideration. The intercept ($\approx 2 \times 10^{-6}$) of the plot in Fig. 2, Part II, would equal K_i in either case, but the slope (≈ 0.11) would equal K_i/K_s (Mechanism Ba) or K_i/K_{ss} (Mechanism Bb). Since $K_s = 2 \times 10^{-5}$ and $K_{ss} = 10^{-3}$, Mechanism Ba would be the correct one.

DISCUSSION

Although compliance with the kinetics of Mechanism Ba is not unequivocal proof of the correctness of this mechanism, it would appear, never-

[4] To be published.

theless, that Mechanism A is incorrect. The results show, furthermore, that the exceptional behavior of xanthine oxidase with respect to the substrate concentration can be interpreted on the basis of the Michaelis theory of enzyme action. The introduction of another principle of action, *e.g.* a chain reaction theory (2), is not necessary.

Since the proposed mechanism is essentially non-competitive, the binding of the 2 xanthine molecules would occur independently at distinct groups on the enzyme surface. In the active complex *ES* the second (auxiliary) group would not take part in the binding of the substrate. Yet, this group would be essential for activity to occur. As in any other form of non-competitive reversible inhibition, this would present a paradox, since there must be interaction between the substrate and this group. It has been proposed (11, 12) that such interaction (electron transport) between distant groups might occur through the protein.

It is of interest to note that the results indicate that, although isoxanthopterin competes with the substrate for attachment to the active group proper, probably because of similarity in structure, no such competition does occur at the "auxiliary group."

Xanthine oxidase activity is known to be dependent on the presence of auxiliary factors, *e.g.* flavin adenine dinucleotide (13). It would be tempting to postulate that the auxiliary group might be the locus of one of these factors. The substrate, aside from combining with the active group proper, might then compete with the prosthetic group for attachment to the enzyme, or prevent the electron acceptor (oxygen) from combining with it.

SUMMARY

The inhibition of xanthine oxidase by its substrate (xanthine) has been interpreted on the basis of the Michaelis theory of enzyme action with the aid of a competitive inhibitor (isoxanthopterin).

The kinetics of the system with respect to varying concentrations of xanthine and isoxanthopterin appear to exclude a competitive multiple point attachment of 2 substrate molecules to the same active group and indicate a non-competitive type of inhibition.

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THE SOURCE AND STATE OF THE HYDROXYLYSINE OF COLLAGEN*

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An unusual feature of the proteins of the collagen group is the presence of the amino acid, 5-hydroxylysine (1–4). Since hydroxylysine is not an essential dietary amino acid (5), it must be synthesized from some precursor either by the addition of a hydroxyl group to the carbon chain of lysine, or by building up the structure of hydroxylysine from small units. The first possibility was investigated by feeding young growing rats L-lysine uniformly labeled with C^{14}. When the lysine and hydroxylysine of the hydrolyzed skin collagen were isolated, it was found that these two amino acids had approximately the same specific activity. Furthermore, the distribution of C^{14} along the carbon chain did not seem to have been altered in the apparent conversion of lysine into hydroxylysine.

EXPERIMENTAL

In a typical experiment a 70 gm. rat was fed 1/12 of a mc. of uniformly C^{14}-labeled L-lysine.[1] This lysine was incorporated at a 1 per cent level in a purified diet (6). The animal was maintained on this diet for a period of 3 weeks prior to sacrifice, at the end of which time it weighed approximately 100 gm. The skin was removed and freed of hair by plucking. It was then cut into small pieces. The pieces were soaked overnight in a mixture of 2 parts of chloroform and 1 part of methanol to extract lipides and were then soaked in 20 per cent urea for 48 hours to remove some of the non-collagenous proteins. The urea was removed by three successive soakings in water. The pieces were then boiled under reflux for 24 hours with 150 ml. of water in order to extract the collagen as gelatin. The solution of gelatin obtained by filtration was concentrated to 25 ml. *in vacuo*. 150 ml. of acetone and 4 ml. of 1 M sodium chloride were added, and on standing overnight a precipitate consisting of 850 mg. of gelatin was obtained. The gelatin was hydrolyzed by refluxing with 6 N HCl for 24 hours.

The hexone bases, lysine, hydroxylysine, histidine, and arginine, were

* This research was supported by the Atomic Energy Commission and Eli Lilly and Company.

[1] Purchased from the Schwarz Laboratories, Inc., 230 Washington Street, Mt. Vernon, New York.

precipitated with phosphotungstic acid according to the procedure of Van Slyke, Hiller, and MacFadyen (2).[2] The phosphotungstates were recrystallized, and the phosphotungstic acid was removed by extraction with amyl alcohol-ether, as described by Van Slyke (9), leaving the hexone bases in a solution of 0.25 N hydrochloric acid. The HCl was removed by distillation *in vacuo*. The residue was dissolved in citrate buffer, and the solution was passed through a Dowex 50 ion exchange column prepared in the sodium phase and operated according to the procedure of Moore and Stein (10). A column 20 cm. in length and 4 cm. in diameter was em-

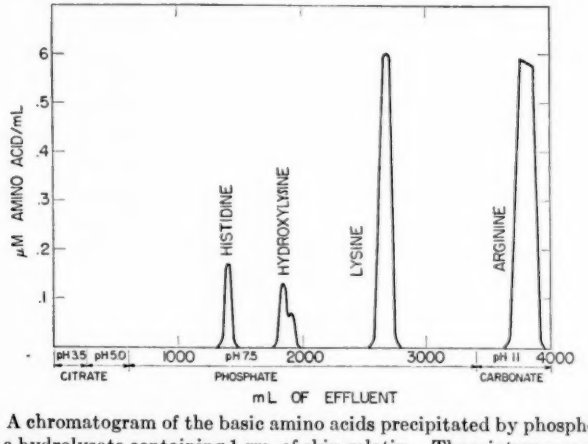

FIG. 1. A chromatogram of the basic amino acids precipitated by phosphotungstic acid from a hydrolysate containing 1 gm. of skin gelatin. The mixture was separated on a 20 × 4 cm. column of Dowex 50 resin with the indicated buffers as eluents.

ployed. Aliquots of 15 ml. were received into each collection tube with the aid of a drop counter.

In order to determine the amount of amino acid in each tube, a 1 ml. aliquot was removed and an analysis by the photometric ninhydrin procedure of Moore and Stein (11) was carried out. The order of emergence

[2] The phospho-12-tungstic acid ($H_3PO_4 \cdot 12WO_3 \cdot 7H_2O$) was prepared from commercial phosphotungstic acid by an application of the ether extraction method that was apparently first used by Drechsel (7). The commercial product was dissolved in a separatory funnel in an equal weight of water, and 1 ml. of concentrated HCl was added for each 10 ml. of water. Ether was then added until three layers of liquid were formed. The bottom layer contained the phospho-12-tungstic acid, the middle layer other phosphotungstic acids, and the top layer excess ether. The bottom layer was washed three times by shaking with equal volumes of water and was dried on a steam bath. The procedure differs from that described in a previous paper (8) in the addition of HCl. Some present commercial preparations of phosphotungstic acid do not form the heavy ether layer unless the HCl is added.

of the hexone bases in phosphate buffer at pH 7.5 is shown in Fig. 1. Histidine emerges first, followed by hydroxylysine, lysine, and arginine. The respective peaks were identified by determining their location with a known mixture of the amino acids. The hydroxylysine peak may also be identified with ninhydrin under the conditions described by Chinard (12) by determining the absorption spectrum of the yellow pigment which is formed. The hydroxylysine showed a characteristic double peak, which has been observed by Hamilton and Anderson (13) and by Piez (14). This double peak indicates the presence of two diastereoisomers of hydroxylysine, one of which is formed by isomerization of the natural isomer during the course of acid hydrolysis as shown by Hamilton and Anderson (13).

The contents of tubes from the centers of the hydroxylysine and lysine peaks, as well as tubes from the other peaks and intermediate valleys, were then analyzed for total C^{14} and for C^{14} in the carboxyl groups of the amino acids.

For determination of the C^{14} activity of the total carbon of the hydroxylysine, aliquots of 2 ml. of each 15 ml. solution in the selected peak were evaporated to dryness, and the total carbon of the amino acids was converted to CO_2 by the wet combustion procedure of Van Slyke *et al.* (15, 16). The $C^{14}O_2$ present was determined by the gas phase proportional counting procedure of Van Slyke, Steele, and Plazin (17). From the disintegrations per minute found and the previous photometric determination of the micromoles of amino acid present, the number of disintegrations per minute per microatom of amino acid carbon was calculated. (The amount of amino acid present was based on the photometric determination rather than on the total estimation of CO_2 yielded by the combustion, because the small amounts of amino acid and the difficulty of complete exclusion of carbon from the reagents made the carbon a less accurate measure of the amount of amino acid.) In order to minimize the carbon of the reagents both BRIJ (a detergent recommended by Moore and Stein) and Versene were omitted from the phosphate buffer at pH 7.5.

For determination of the C^{14} activity of the carbon in the carboxyl groups, duplicate portions of 4 ml. were treated with ninhydrin by the procedure of Van Slyke, Dillon, MacFadyen, and Hamilton (18), and the amount of C^{14} in the evolved carbon dioxide was determined by counting in the gas phase (17). In calculating disintegrations per minute per microatom of carboxyl carbon, the latter value was again estimated from the photometrically determined hydroxylysine, since the amount of CO_2 released by ninhydrin was not sufficient for accurate manometric measurement.

The experiment was repeated with five successive rats, with gradually improved technique. Data from the fifth rat are shown in Table I. The

specific activities of the total carbon of hydroxylysine and lysine are almost identical, indicating that hydroxylysine in the collagen was formed from C^{14}-labeled lysine that had been fed. Furthermore, it would appear that the hydroxylation of the lysine occurred without any changes in the carbon chain, since hydroxylysine, like the lysine, had the same specific activity in the carbon of the total chain as in the carboxyl carbon. Substitution of C^{12} in either the carboxyl or the rest of the chain would have caused one or the other to have lower specific activity.

Non-Existence of Phosphorylated Hydroxylysine in Collagen—Gordon (19) reported the presence of the phosphoric acid ester of hydroxylysine in calf embryo juice, and Astrup, Carlström, and Stage (20) found it present in the liver of the adult ox. Since Lipmann (21) and Levene and Hill (22) have shown that the hydroxyl group of serine in casein is esterified with phosphoric acid, the question of whether the hydroxylysine in collagen is

TABLE I

Specific Activity of Carbon Atoms of Lysine and Hydroxylysine Obtained from Skin Collagen after Feeding L-*Lysine Uniformly Labeled with* C^{14}

Amino acid	Disintegrations per min. per microatom carbon	
	Total carbon by combustion	Carboxyl carbon by reaction with ninhydrin
Hydroxylysine	363	349
Lysine	365	372

phosphorylated appeared pertinent. Desnuelle and Antonin's (4) finding that treatment of gelatin with periodate liberates a molecule of formaldehyde per molecule of hydroxylysine present in the protein made esterification of the hydroxyl group in gelatin appear improbable, but the possibilities were not excluded that the formaldehyde might be formed by action of the periodate on a small amount of carbohydrate in the protein molecule, or that phosphoric acid esterified with the hydroxylysine in collagen might be split off during the transformation of collagen into gelatin, which includes prolonged boiling with water, and in some technical productions treatment with calcium hydroxide.

Determinations of total phosphorus in native collagen showed that the amounts present were insufficient to esterify more than a small fraction of hydroxylysine. The tail tendons of a dog were dissected free of accompanying fascia and washed in isotonic saline. They were then dried to constant weight. Samples of 8 to 10 mg. of this material were subjected to digestion with perchloric acid and photometric phosphorus determination by the method of King (23). The phosphorus present was 0.22 mg.

per gm. of dry tendon. Assuming, as found in several analyses (1–4), the hydroxylysine nitrogen to be approximately 1 per cent of the collagen nitrogen, and that the tendon was predominantly collagen, a phosphorus content of approximately 2.3 mg. per gm. of collagen would be required to esterify the δ-hydroxyl group of the hydroxylysine.

DISCUSSION

Hydroxyproline, the other amino acid that is characteristic of collagen, presents an interesting analogy. Stetten and Schoenheimer (24) fed labeled proline to rats for 3 days and estimated that about one-quarter of the total hydroxyproline in the carcass at the end of that period had been formed from the administered proline. The conversion did not appear to be reversible, since when Stetten (25) fed labeled hydroxyproline the carcass showed no more than traces of either proline or hydroxyproline in the labeled form. Stetten concluded that the hydroxyproline of the body proteins "is not derived to any appreciable extent from dietary hydroxyproline but rather from oxidation of the proline which is already bound, presumably in peptide linkage." In later experiments (26) Stetten found that N^{15}-labeled ornithine could serve as a source of both proline and hydroxyproline; however, the N^{15} concentration of the hydroxyproline was only one-third as high as in the proline, an effect consistent with the view that proline is a precursor of hydroxyproline.

It appears possible that the relations of lysine and hydroxylysine may parallel those of proline and hydroxyproline. Labeled hydroxylysine has been prepared by the procedure of Lindstedt (27) and will be used to determine whether or not administered hydroxylysine can be incorporated into collagen either directly as hydroxylysine or after removal of the hydroxyl group as lysine.

For excellent technical assistance we thank Esther Hollister, Mary Phillips, and Herbert Schulman.[3]

SUMMARY

In order to find whether hydroxylysine is formed from lysine in the animal body, young rats were fed for 3 weeks L-lysine uniformly labeled with C^{14}. The rats were then sacrificed, and gelatin was prepared from the skin collagen and hydrolyzed. The basic amino acids were separated from the hydrolysate by precipitation with phosphotungstic acid and then from each other by means of a Dowex 50 ion exchange column. The C^{14} specific activity was the same in the hydroxylysine as in the lysine, and in

[3] Mr. Schulman was a student appointee at Brookhaven while participating in the student field training program of Bard College.

both of the amino acids the specific activity was the same in the carboxyl group as in the rest of the 6-carbon chain, indicating the probability that the uniform C^{14} labeling of the fed lysine was retained.

The results indicate that hydroxylysine in the body is formed from lysine. The approximate equality of C^{14} activity in both amino acids indicated the probability that but little hydroxylysine is formed from other sources.

Dog tail tendon contained only a small fraction of the amount of phosphorus that would be present if the hydroxyl group of the hydroxylysine were esterified with phosphoric acid.

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THE ACCUMULATION OF KETO ACIDS AND ACETALDEHYDE BY A STRAIN OF NEUROSPORA INHIBITED BY THREONINE*

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In the course of investigations into the nature of the threonine inhibition of a strain of *Neurospora crassa*, T77, several facts of biochemical interest were discovered which relate to the metabolism of threonine. T77 is a single gene mutant strain of *Neurospora* which grows well on minimal medium, but is strongly inhibited by L-threonine, giving only 10 mg. of growth in 3 days in the presence of L-threonine as opposed to about 55 mg. in its absence (1, 2). The inhibition is strongly expressed only at 35° and for approximately 3 days after inoculation. After the 3rd day the mutant grows almost as rapidly as the wild type strains.

An analysis of the metabolic products excreted into the medium by this strain when growing in the presence of threonine revealed that three keto acids, α-keto-β-methylvaleric, α-ketoisovaleric and pyruvic, and acetaldehyde are accumulated. This accumulation does not occur when the mutant is growing on minimal medium, and it is accompanied by a decrease in the threonine concentration.

This communication deals with the isolation and identification of these compounds and the bearing of these data on the knowledge of the metabolism of threonine by *Neurospora*. In addition, quantitative estimates of the amount of keto compounds accumulated during the course of growth are given. Comparative data are presented for both the mutant, T77, and the wild type strain, Em5256, used in the experiments.

EXPERIMENTAL

Methods—15 liters of *Neurospora* minimal medium (3) were supplemented with 10 gm. of DL-threonine, inoculated with T77, incubated at 37°, and aerated continuously for 4 days. On the 2nd day after inoculation the air exit from the culture container was attached by a rubber hose to a sintered glass bubbler immersed in a 0.01 M solution of 2,4-dinitrophenylhydrazine

* These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and The University of Texas, NR859(00), and a research grant (C-2269) from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

in 2 N HCl. Hence all air passed through the culture medium during the 3rd and 4th days of growth was also secondarily bubbled through the phenylhydrazine reagent.

At the end of the 4th day of growth the crystals of hydrazone which had accumulated on the bubbler surface were recovered. The mold mycelium was removed from the culture medium by filtration, and to the medium were added 2 liters of a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl. The mixture was filtered after standing overnight and the precipitate dried in air.

The filtered medium was analyzed for the presence of amino acids by two-dimensional paper chromatography with water-saturated phenol and 2,4-lutidine as the solvents and Whatman No. 1 filter paper. Paper chromatography of the dinitrophenylhydrazones was carried out with a solvent composed of *n*-butanol, ammonia, and water prepared by saturating *n*-butanol with a 10 per cent solution of aqueous ammonia.

Adsorption column chromatography of the hydrazones was accomplished with dry, activated alumina, 20 mesh.

The x-ray diffraction tracings were obtained with a high angle Norleco diffractometer with Cu K radiation.[1]

Acetaldehyde—The crystalline material removed from the sintered glass bubbler was washed with 2 N HCl and dried *in vacuo*. The crystals were identified as form I (4) of the 2,4-dinitrophenylhydrazone of acetaldehyde by x-ray diffraction. The x-ray diffraction powder pattern of the isolated hydrazone and that for an authentic sample as determined by Gordon *et al.* (4) are reported in Table I.

The melting point of the isolated dinitrophenyldrazone was 154°. The melting point was not depressed when the crystals were mixed with an authentic sample of acetaldehyde 2,4-dinitrophenylhydrazone.

The wild type strain, Em5256, was also demonstrated to produce acetaldehyde in the presence of threonine by this method. Neither the wild type nor the mutant produced a detectable amount of acetaldehyde, however, in the absence of threonine.

α-Keto-β-methylvaleric Acid—An activated alumina chromatographic column was prepared with anhydrous ethyl acetate as the liquid component. The dimensions of the column were 45 × 2.2 cm. The precipitate from the medium (total dry weight, 2.20 gm.) was taken up in a minimal amount of anhydrous ethyl acetate and added to the column. The continued addition of ethyl acetate carried a yellow solution through the column which proved to be excess phenylhydrazine reagent and some pyruvic acid hydra-

[1] The x-ray diffraction patterns were made by Dr. Joseph E. Weiss of the Department of Ceramic Engineering of The University of Texas. The authors acknowledge his contribution with thanks.

zone in solution. This effluent was discarded. When the ethyl acetate effluent became colorless, a mixture containing 80 per cent of 95 per cent ethyl alcohol and 20 per cent ethyl acetate was added. The single band at the top of the column separated into two components, one of which remained completely absorbed; the other was eluted. The effluent was reduced to dryness in air. The crystals remaining had a dry weight of 1.08 gm. These were recrystallized from ethyl alcohol, dissolved in water, and twice reprecipitated by the addition of sufficient 1 N HCl to remove the yellow color from the solution. The crystals were then filtered and dried *in vacuo*.

TABLE I

x-Ray Diffraction Powder Pattern of 2,4-Dinitrophenylhydrazones of Acetaldehyde

Form I,* $d(hkl)$	Hydrazone from medium, $d(hkl)$
A	*A*
9.35	9.34
6.75	6.73
5.70	5.69
4.88	4.90
4.71	4.70
3.95	3.94
3.58	3.57
3.23	3.22
3.15	3.18
3.03	3.02

* Gordon *et al.* (4).

The x-ray powder pattern for these crystals is reported in Table II. Table II also contains the pattern for a known sample of the hydrazone of α-keto-β-methylvaleric acid prepared by reprecipitation with HCl. This sample was prepared synthetically and is a racemic mixture of the *d* and *l* forms of α-keto-β-methylvaleric acid.[2]

The melting point for the isolated crystals was 171°, which agrees with the melting point of the known sample and that given by Stumpf and Green (5) for this compound. Meister (6) has shown that a racemic mixture of the 2,4-dinitrophenylhydrazones of the *d* and *l* forms of α-keto-β-methylvaleric acid has a melting point of 168–169°. This is in contrast to the melting point of 176° of the pure *d* and *l* forms. It is concluded therefore that the dinitrophenylhydrazone of the compound isolated from the medium is a racemic mixture.

[2] Obtained through the courtesy of Dr. William Shive, Department of Chemistry, The University of Texas.

The elementary analysis of the purified crystals of the dinitrophenylhydrazone was as follows: calculated for $C_{12}H_{14}O_6N_4$, C 46.4, H 4.5, N 18.1 per cent; found, C 46.2, H 4.2, N 17.6 per cent.

Paper chromatography of the compound with a butanol-ammonia-water solvent gave a single spot of R_F 0.80, which corresponded exactly to the R_F of the known sample in the same solvent. The absorption spectrum of the hydrazone in a solution of 0.75 N NaOH had a peak in the same

TABLE II

x-Ray Diffraction Powder Patterns

2,4-Dinitrophenylhydrazone from T77 medium; eluted with ethyl acetate and ethyl alcohol		2,4-Dinitrophenylhydrazone of α-keto-β-methylvaleric acid	
d(*hkl*)	Relative intensity	*d*(*hkl*)	Relative intensity
A		*A*	
15.1	0.4	15.3	0.5
10.9	1.0	10.9	1.0
7.66		7.69	
6.86	0.5	6.92	0.4
5.51		5.53	
4.90		4.93	
4.27		4.29	
3.73		3.75	
3.65		3.67	
3.46		3.48	
3.35	0.3	3.36	0.5
3.20		3.21	
2.98		3.03	
2.65		2.65	
2.51		2.51	

region reported for the keto analogue of isoleucine by Metzler, Olivard, and Snell (7).

α-Ketoisovaleric Acid—The top band of the column was eluted with 0.05 M potassium phosphate at pH 7.8. The effluent was taken to dryness in air at room temperature. The hydrazone crystals were dissolved in ethyl alcohol, recrystallized, dissolved in water, and twice reprecipitated by the addition of HCl. The crystals were dried *in vacuo*.

The x-ray diffraction powder pattern for these crystals is given in Table III together with the pattern for a known sample of the dinitrophenylhydrazone of α-ketoisovaleric acid.[3]

The melting point of the crystals was 195°. The known sample had a

[3] Obtained through the courtesy of Dr. Esmond Snell, Department of Chemistry, The University of Texas.

melting point of 193°; Waelsch and Miller (8) report 194° for this compound. No depression of melting point was noted on mixing the isolated and known samples.

TABLE III

x-Ray Diffraction Powder Patterns

2,4-Dinitrophenylhydrazone from T77 medium; eluted with phosphate buffer		2,4-Dinitrophenylhydrazone of α-ketoisovaleric acid	
d(*hkl*)	Relative intensity	*d*(*hkl*)	Relative intensity
A		*A*	
15.0	0.3	14.9	0.3
10.5	1.0	10.5	1.0
8.34		8.39	
7.48		7.51	
6.52	0.3	6.51	0.4
5.54		5.54	
5.37		5.36	
5.02		5.02	
4.70		4.67	
4.46		4.43	
4.28		4.29	
4.20		4.20	
4.11		4.11	
3.98		3.98	
3.85		3.83	
3.73		3.72	
3.64		3.64	
3.53		3.52	
3.36	0.7	3.36	0.9
3.21	0.3	3.20	0.4
3.00		3.01	
2.94		2.93	
2.78		2.79	
2.68		2.67	
2.57		2.57	
2.45		2.45	

The elementary analysis of the dinitrophenylhydrazone was as follows: calculated for $C_{11}H_{12}O_6N_4$, C 44.6, H 4.1, N 18.9 per cent; found, C 45.3, H 4.0, N 18.9 per cent.

The R_F of the isolated hydrazone in butanol-ammonia-water solvent was 0.67 and corresponded exactly with the R_F of the dinitrophenylhydrazone of the known sample of α-ketoisovaleric acid. The absorption spectrum showed a peak in the same region that Metzler, Olivard, and Snell (7) found for the keto acid analogue of valine under the same conditions.

Pyruvic Acid—The 2,4-dinitrophenylhydrazone of pyruvic acid moves through an alumina column in a series of bands, some of them diffuse, with the solvents employed in this study. Separation of the pyruvic acid fraction from the other hydrazones was effected therefore by paper chromatography. To 25 ml. of the medium of a 3 day-old culture of strain T77 growing in the presence of an initial concentration of 0.004 M L-threonine were added 10 ml. of 0.01 M 2,4-dinitrophenylhydrazone in 2 N HCl. After formation of the hydrazones the mixture was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was reduced to 2 ml. in volume and 0.05 ml. of this spotted on paper. A butanol-ammonia-water solvent resolved four spots. The two at R_F 0.80 and 0.67 were shown above to be the hydrazones of α-keto-β-methylvaleric acid and α-ketoisovaleric acid respectively. The lower two spots, with an R_F of 0.42 and 0.60, corresponded to the two spots given by a known sample of the dinitrophenylhydrazone of pyruvic acid. The spots were cut out of the paper and eluted with 0.05 M potassium phosphate buffer at pH 7.8. The eluates were diluted to 2 ml. with water, and 2 ml. of 1.5 M NaOH were added. The absorption spectra of the solutions were determined immediately in the Beckman spectrophotometer. The lower spot had an absorption maximum of 445 mμ, the upper spot a maximum of 395 mμ. These two maxima correspond exactly to the maxima found by Metzler, Olivard, and Snell (7) for the slower and faster moving zones of the dinitrophenylhydrazone of pyruvic acid.

Quantitative Estimates of Amino Acid and Keto Acid Content of Medium—Cultures of T77 were grown in 25 ml. of minimal medium supplemented with 100 μmoles of L-threonine. The pH of the medium was adjusted to 5.0, and the temperature of incubation was 37°. Flasks were removed and tested for their threonine and keto acid content every 24 hours. At the same time the accumulated mycelium was removed, brought to dry weight, and weighed. An aliquot of the medium from each flask was assayed for its L-threonine content with *Streptococcus faecalis* as the assay organism according to the method of Wiss (9). 10 ml. of 0.01 M 2,4-dinitrophenylhydrazine in 2 N HCl were added to the remainder of the medium. The hydrazones were extracted with sufficient ethyl acetate to remove all traces of yellow color from the aqueous phase. The ethyl acetate extracts from each flask were then chromatogramed by the method discussed above for pyruvic acid. The spots were cut out of the paper, the hydrazones eluted, and the extinction values determined at 432 mμ in 0.75 M NaOH. Only the lower spot of the pyruvic acid dinitrophenylhydrazone was used in these determinations. Standard calibration curves for the dinitrophenylhydrazones of α-ketoisovaleric acid, α-keto-β-methylvaleric acid, and pyruvic acid were prepared at the 432 mμ wave-length, and the extinction coeffi-

cients of the samples were converted to micromoles per 25 ml. by the use of these curves.

The results for the keto acid concentrations in flasks from 1 to 5 days after inoculation are presented in Table IV, Experiment 1. The last column records values for the threonine determinations made for the same flasks. Experiments 2 and 3 were carried out precisely as described above and with the same analytical procedure, except that a larger inoculum of T77 was used in order to obtain a greater amount of mycelium in a shorter period of time. Data are not included for the first 2 days of growth, since

TABLE IV

Quantitative Estimates of Keto Acids Accumulated in Medium of T77 While Growing in Presence of L-*Threonine*

The results are expressed in micromoles per 25 ml.

Experiment No.	Growth period	Dry weight of mycelium	α-Keto-β-methyl-valeric acid	α-Ketoiso-valeric acid	Pyruvic acid	Total keto acids	L-Threonine
	days	*mg.*					
1	1	Trace	0	0	0	0	95
	2	1	0	0	0	0	97
	3	13	0	0	3.8	3.8	95
	4	24	8.5	1.8	11.1	21.4	16
	5	45	5.2	0.8	5.6	12.0	2.3
2	3	26	6.9	3.0	9.0	18.9	4.2
	4	38	8.0	4.8	4.9	17.7	2.2
	5	44	6.7	1.3	2.7	10.7	1.0
3	3	32	7.8	9.1	5.0	21.9	
	4	38	9.5	6.9	4.4	20.8	
	5	53	2.5	2.1	1.7	6.3	

keto acid accumulation before the 3rd day is negligible. The use of a large inoculum does not increase the total keto acid production, it will be noted, but it does result in an increased amount of α-ketoisovaleric acid.

It will be noted from Table IV that the appearance of the keto acids is accompanied by a concomitant decrease in L-threonine. In order to check the validity of the assay results obtained with *S. faecalis*, paper chromatograms were made with aliquots of the same medium that was assayed microbiologically in Experiment 1. It could be seen from these that the threonine spot (which was the only one to be noted) was weakened after the 3rd day of growth, finally disappearing on the 5th day, in complete agreement with the microbioassay. The three keto acids were tested for their effect on the growth of the assay organism. None was found.

Control experiments in which T77 was grown in unsupplemented mini-

mal medium showed that it produces none of these keto acids in the absence of L-threonine at any time during the course of its growth, except for trace amounts of pyruvic acid which appear transitorily during the 2nd day of growth.

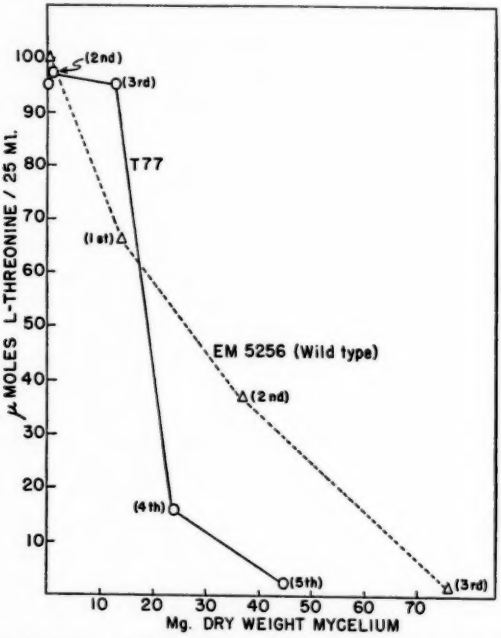

FIG. 1. Loss of L-threonine from medium of T77 and wild type as a function of dry weight of accumulated mycelium. The age of the mycelium in days is indicated for each point on the graph except for the 1st day's growth of T77, which was less than 1 mg.

The production of acetaldehyde by *Neurospora* in the presence of threonine would suggest that threonine is cleaved between the α- and β-carbons, and hence glycine would be expected to be produced. Two-dimensional paper chromatograms revealed that glycine as well as threonine was present in the medium in accordance with the prediction. However, glycine was also found in the medium of T77 growing in the absence of threonine, and in the medium of wild type, both in the presence and absence of threonine. Quantitative determinations of the glycine in the medium after various periods of growth, made by microbiological assay with *Leuconostoc mesenteroides* by the method of Wiss (9), showed that the amounts of glycine

accumulated by both strains in the presence or absence of threonine were in all cases approximately the same.

Comparison of T77 and Wild Type—Threonine does not inhibit the growth of wild type, nor does wild type accumulate keto acids except for small amounts of pyruvate when growing in the presence of threonine. The wild type does produce acetaldehyde when threonine is present, as discussed above, and in this respect it is similar to the mutant. Threonine is rapidly utilized by wild type during the course of its growth, as is shown by Fig. 1 in which the amount of threonine present in the medium, determined at daily intervals, is plotted against the dry weight of the mycelium accumulated up to the time of determination. Fig. 1 also includes the data for threonine concentrations reported in Table IV, Experiment 1, plotted against the mycelial dry weight values. It will be noted that the disappearance of threonine in the presence of T77 is more rapid than in the presence of wild type, despite the more rapid growth rate of wild type.

DISCUSSION

The interest in T77 as a biochemical mutant lies in the facts that, although it grows well on minimal medium at 35°, it is inhibited strongly by L-threonine, and that the threonine inhibition is completely relieved by methionine, homocysteine, or homoserine, or a combination of choline and thiazol (1, 2). The fact, now demonstrated, that the mutant also accumulates three keto acids, but only in the presence of threonine, suggests that the inhibitory effect of threonine is in some way related to the accumulation of these acids. This question cannot be resolved on the basis of the present data, since the accumulation may either be the direct result of threonine inhibiting the utilization of the keto acids or the overproduction of them by direct conversion from threonine, as discussed below, resulting in a metabolic imbalance which causes inhibition of growth. Another alternative is that threonine may be both acting as a precursor of one or more of the keto acids and, in addition, causing an inhibition of their further conversion.

Preliminary unpublished experiments have shown that the accumulation of the keto acids by T77 is greatly reduced in the presence of the amino acids and other compounds which relieve the inhibition of threonine. This is particularly true for α-ketoisovaleric acid which, under these conditions, appears only transitorily on the 3rd day of growth in trace amounts. These results are difficult to interpret, however, since the experiments were carried out with growing cultures.

The nature of the metabolic dysfunction in strain T77 which causes the sensitivity to threonine and the accumulation of α-keto-β-methylvaleric, α-ketoisovaleric, and pyruvic acids is unknown, but the accumulation of

these compounds is of considerable present interest, particularly when taken together with the finding that acetaldehyde is also formed in the presence of threonine.

Previous work reported by Umbarger and Adelberg (10) indicated that valine and isoleucine are synthesized from threonine via the condensation of the keto acid derivative of threonine, α-keto-β-hydroxybutyric acid, and acetate or a related 2-carbon unit. More recently, by the use of C^{14}-labeled lactate, acetate, glycine, and glucose supplied to the yeast *Torulopsis utilis*, Strassman, Thomas, and Weinhouse (11) obtained results which could be explained on the basis that valine is formed via an aldol condensation between acetaldehyde and pyruvic acid. They postulated that the condensation product then undergoes a pinacol rearrangement to form α-keto-isovaleric acid. Their data also indicated that acetate and glycine incorporated into valine do so only after prior conversion to pyruvate.

Adelberg (12) and Strassman, Thomas, Locke, and Weinhouse (13) have presented data, based on the use of C^{14}-labeled compounds, which make it evident that isoleucine is formed from threonine via an aldol condensation of acetaldehyde and α-ketobutyric acid. According to the results obtained by Adelberg (12) with a mutant of *Neurospora*, this condensation is followed by a pinacol rearrangement which results first in the formation of the dihydroxy precursor of α-keto-β-methylvaleric acid, which is then converted to the keto acid precursor of isoleucine (14). Thus the same type of biosynthetic pathway is postulated for the formation of both amino acids, valine and isoleucine.

The formation of acetaldehyde from threonine by *Neurospora* indicated by the results reported here extends the known occurrence of the cleavage of threonine to yield glycine and acetaldehyde. It has already been established by the use of liver preparations that it occurs in mammalian tissue (15, 16). Threonine can also be cleaved catalytically in the presence of pyridoxal and metal salts (17) in the absence of any cellular constituents. Tracer work employing threonine labeled with C^{14} and N^{15} has indicated that the fate of this amino acid in the mammal is degradation to acetic acid, presumably via acetaldehyde, and glycine (18).

The results reported in this communication are in agreement with the demonstration of Adelberg (12) that threonine is a precursor of isoleucine in *Neurospora*, since the keto acid precursor of isoleucine is accumulated only in the presence of threonine, and since threonine disappears rapidly from the medium during the period of accumulation of the keto acid. These data may be interpreted alternatively to mean that the α-keto-β-methylvaleric acid accumulates because of threonine inhibition only and that threonine does not directly enter into its formation. The concomitant disappearance of threonine may only indicate that it is being used in metab-

olism elsewhere. Work with labeled threonine or isolated enzyme systems would be the only way to resolve this question with certainty for T77, but the fact that tracer studies by Adelberg (12, 19) with another *Neurospora* mutant do show conclusively that threonine contributes to isoleucine formation makes the first interpretation stated above the more likely one in the absence of further data.

The production of acetaldehyde by *Neurospora* in the presence of threonine leads to the possibility that threonine contributes to all 6 carbons of isoleucine. It has already been shown that *Neurospora* synthesizes α-ketobutyric acid from threonine by a dehydrase (20). The presence of a similar enzyme in T77 has been verified in this laboratory. If it is assumed that the disappearance of threonine indicates that threonine is being converted to α-keto-β-methylvalerate, then it may also be postulated that this conversion is being effected by the aldol condensation of acetaldehyde and α-ketobutyrate derived from threonine. This conclusion is not directly supported by the results of Adelberg (12), who showed that, when *Neurospora* resting mycelial pads were incubated with acetate labeled in both carbons and unlabeled threonine, the labeled carbons appeared in the 3 and 6 positions of the dihydroxy precursor of isoleucine. However, if acetaldehyde is produced from threonine under the conditions of active growth described in this communication, it is difficult to avoid the conclusion that it may be contributing to the pool of 2-carbon units which is presumably utilized in the biosynthesis of isoleucine.

The accumulation of pyruvic acid and α-ketoisovaleric acid in the presence of threonine may be taken to indicate that α-ketoisovaleric acid is also derived from threonine via the condensation of acetaldehyde and pyruvate derived from threonine. Pyruvic acid may be postulated to be derived from the serine formed from glycine (21, 22) resulting from the cleavage of threonine. However, Adelberg (19) has shown that when threonine labeled at carbons 1 and 2 with C^{14} is fed to *Neurospora* mutant 35423-16117, which accumulates α,β-dihydroxymethylvaleric acid and α,β-dihydroxyisovaleric acid, the dihydroxy precursor of isoleucine, but not of valine, is labeled at the α- and carboxyl carbons. Although these results (19) do not preclude the possibility of acetaldehyde from threonine participating in valine biosynthesis, they do demonstrate that glycine from threonine probably does not play a direct rôle in valine biosynthesis in the *Neurospora* strain used by Adelberg in his experiments or in wild type *Neurospora*. It is possible, however, that T77 may have a metabolism different from 35423-16117 and produce valine from pyruvic acid and acetaldehyde derived from threonine. None of these points can be established decisively in T77 until the necessary experiments with tracers and the isolated enzyme system are carried out. Work on the enzymes involved is now being done in this laboratory.

SUMMARY

1. *Neurospora crassa*, strain T77, accumulates in its medium when growing in the presence of threonine, α-keto-β-methylvaleric acid, α-ketoisovaleric acid, pyruvic acid, and acetaldehyde. These compounds are not accumulated when this strain grows in the absence of threonine, nor are they, with the exception of acetaldehyde, accumulated by wild type in the presence of threonine.

2. Evidence for the nature of the compounds accumulated includes quantitative analysis, x-ray diffraction patterns, paper chromatography, and absorption spectra of the 2,4-dinitrophenylhydrazones.

3. The accumulation of the keto acids and acetaldehyde is accompanied by a disappearance of threonine in the mutant strain.

4. The significance of these findings with respect to the metabolism of threonine, isoleucine, and valine is discussed.

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STUDIES ON HYALURONIC ACID IN THE VITREOUS BODY*

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Hyaluronic acid from umbilical cord, according to earlier light scattering studies (1), has a molecular weight of 3 to 4 $\times$ 10^6, and the scattering envelope fits that of a somewhat stiff coil. There are indications that the molecular characteristics of hyaluronic acid may vary according to the source. Hyaluronic acid from the vitreous body has always been reported to have a much lower viscosity than hyaluronic acid from umbilical cord (2, 3). This report deals with a light scattering and viscosity study of sodium hyaluronate from the vitreous body. In order to obtain a preparation of hyaluronic acid by as mild a procedure as possible, a new method of preparation has been worked out.

Methods

Preparation—About 200 fresh cattle eyes were cleaned from surrounding tissues and washed with water. After the globes were divided into two parts by an equatorial cut, the posterior parts of the vitreous gel were taken out. The gel pieces were carefully cleaned from adhering retinal tissue and were then dialyzed under stirring against several changes of 4 liter portions of distilled water for 24 hours at 5°. The dialysis was continued at 5° for 12 hours against a 4 liter agitated suspension of 200 gm. of Dowex 50 cation exchange resin (H cycle, 200 to 400 mesh, 8 per cent cross-linking). During the deionizing procedure a white precipitate was formed consisting of large flakes, one for each piece of vitreous gel. The pH of the solution dropped to 3.0 to 3.5. In certain cases, when the dialysis against distilled water had not been complete, the pH dropped below 3.0. These solutions were discarded. The solution was centrifuged for 15 minutes at 45,000 $\times$ g and the supernatant solution was collected (Fraction A). This fraction was concentrated to 25 to 50 ml. by lyophilization and to this solution sodium chloride was added to a final con-

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centration of 6 N. A fine precipitate appeared. The solution was shaken for 1 hour and then centrifuged at 45,000 × *g* for 5 hours at 1–4° when all the fine precipitate sedimented. In order to remove the sodium chloride, the supernatant solution was dialyzed against distilled water. This was followed by dialysis against a resin suspension, under the same conditions described above (Fraction B).

Fraction B was neutralized with 0.02 N sodium hydroxide; the neutral end-point was checked with a glass electrode, and then lyophilized. The amount of sodium hydroxide needed for the neutralization was equivalent to the glucosamine content. The methods used for analysis have been

TABLE I

Analytical Values in Per Cent of Dry Weight of Fraction B (As Sodium Salt) Compared with Theoretical Values for Sodium Hyaluronate $(C_{14}O_{11}H_{20}NNa)_n$

	Per cent of dry weight of preparation	Theoretical values	Method used for analysis (bibliographic reference Nos.)
		per cent	
Glucosamine	37.5	44.6	(4)
Galactosamine	1.7		(4)
Hexuronic acid	43.5	48.6	(5)
Carbon	38.0	41.9	(6)
Hydrogen	5.2	5.0	(6)
Nitrogen	3.9	3.5	(7)
Ash	4.9	5.7	
Sulfur	0.08		(8)
Sodium	5.0	5.7	(9)

described previously (1). The total hexosamine content of the starting material was 290 mg. per 1 liter of vitreous gel. The non-hexosamine nitrogen content was 112 mg. per 1 liter of vitreous gel. In Fraction A the total hexosamine content was 185 mg. and the non-hexosamine nitrogen was 17.2 mg. per 1 liter, respectively. In Fraction B, total hexosamine was 140 mg. and non-hexosamine nitrogen was 3.1 mg. per 1 liter, respectively. These determinations seem to indicate that about one-half of the total hyaluronic acid of the vitreous body is collected in Fraction B with a probable protein impurity of 5.3 per cent (non-hexosamine nitrogen × 6.25). The data obtained from a more complete analysis of a large batch of Fraction B are presented in Table I.

Light Scattering Measurements—For the light scattering experiments, 0.3 to 1.0 per cent of Fraction B was dissolved in dust-free (see below) distilled water, and the ionic strength was adjusted by adding 2 N sodium

chloride. All dilutions were then made with sodium chloride solutions of the same ionic strength. The pH, measured on a Beckman model G pH meter with a glass electrode, was always between 5.5 and 6.5. Concentration determinations were based upon hexosamine analysis (1).

Light scattering was measured at angles from 25° to 135° in a Brice-Speiser light scattering apparatus as described earlier (1). The light used was unpolarized of a wave-length of 4360 A. 4.58×10^{-7} was used as the value of the constant K ($K = 2\pi^2 n^2 (dn/dc)^2 / N\lambda^4$).[1] The results were evaluated according to Zimm (10).

To remove dust, the solutions were first filtered through a Millipore filter[2] (90 per cent of the hexosamine was filterable). As this was not sufficient to remove larger particles (see "Results"), they were then centrifuged for at least 12 hours at $50{,}000 \times g$ in a Spinco preparative ultracentrifuge at 4°. The solutions were pipetted into conical cells containing filtered sodium chloride solutions of the same ionic strength as the sodium hyaluronate solutions. The pipetting was made in four stages and the scattering envelope was measured at four different concentrations.

Viscometry—A somewhat modified Ubbelohde viscometer was used (11). The horizontal capillary had a length of 8.25 cm. and a diameter of 0.05 cm. There were four bulbs and their volumes were 1.00, 1.00, 0.75, and 0.50 ml., respectively. The mean top levels of the bulbs were calculated according to Meissner's formula (11) to be 10.8, 8.2, 5.9, and 3.8 cm., respectively. The measurements were carried out at 20°. The outflow time of water for the different bulbs was 37.0, 47.3, 48.3, and 51.9 seconds, respectively. The kinetic errors for the different bulbs were $41/t$, $62/t$, $59/t$, and $55/t$, respectively (12). The average velocity gradient was calculated according to Kroeplin's formula (13) to be $54{,}400/t$, $54{,}400/t$, $40{,}600/t$, and $27{,}000/t$ sec.$^{-1}$, respectively.

The solutions to be measured were first centrifuged for 30 minutes at $20{,}000 \times g$ to remove dust that could interfere with the measurements. 6 ml. were then pipetted into the viscometer and the outflow time was measured with an accuracy of 0.2 second. From the values for the four bulbs it was possible to make a rectilinear extrapolation to zero velocity gradient.

Results

Light Scattering—Measurements were first performed on Fraction B after filtration through a Millipore filter. The filtering procedure is sufficient to remove all dust from the buffer solutions. Calculation of the

[1] n is the refractive index of the solvent; dn/dc, the refractive increment; N, Avogadro's number; λ, the wave-length of the light.

[2] Millipore Filter Corporation, Watertown, Massachusetts.

molecular weight and shape indicated that the molecule was a random coil with a molecular weight of 1.3×10^6. To check the presence of any impurity with a considerably larger molecular weight, the measurements were repeated after centrifugation of the solutions.

0.3 to 0.5 per cent solutions of Preparation B containing sodium chloride, ionic strength 0.1, were centrifuged for 12, 24, and 36 hours at 50,000 × *g*. The concentrations were checked afterwards and it was found that the

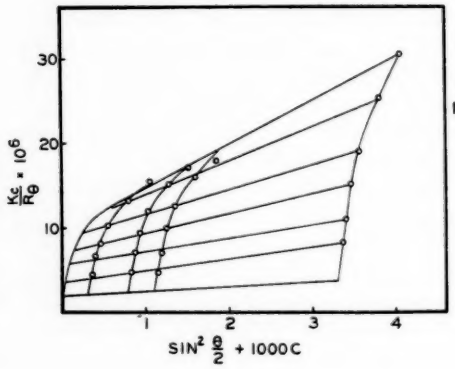

FIG. 1

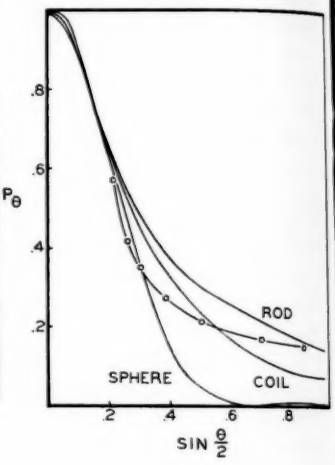

FIG. 2

FIG. 1. Zimm plot for hyaluronic acid centrifuged for 20 hours. pH 6.0. Ionic strength 0.10. Calculated molecular weight 500,000. Root-mean-square radius 1970 A.

FIG. 2. Comparison of the scattering envelope for hyaluronic acid from vitreous humor with theoretical curves for a rod, a random coil, and a sphere with a root-mean-square radius of 1950 A.

amount of non-hexosamine nitrogen had decreased by 30 per cent in the supernatant solutions. The same Zimm plot was obtained after any of the three centrifugations. A typical Zimm plot after centrifugation is shown in Fig. 1. The molecular weight varied between 340,000 and 500,000 in six different determinations. The root-mean-square radius (10), a parameter related to the length of the molecule (calculated from the initial slope of the scattering envelope at zero concentration in the plot), was 1900 to 2000 A.

If one knows the shape of the molecule, it would be possible to calculate the actual length from the root-mean-square radius. Theoretical scattering envelopes have been calculated for rods, random coils, and spheres (14).

The scattering envelope for the centrifuged Fraction B is represented in Fig. 2 and compared with the theoretical functions of the same root-mean-square radius. It fits neither of these three possibilities nor the scattering envelopes for ellipsoids and cylinders given by Saitô and Ikeda (15).

The shape of the scattering envelope can be explained on the basis of a multicomponent system. Either there are two or more macromolecular substances present in Fraction B or the hyaluronic acid is highly polydisperse, the large particles being more kinked and the smaller ones being

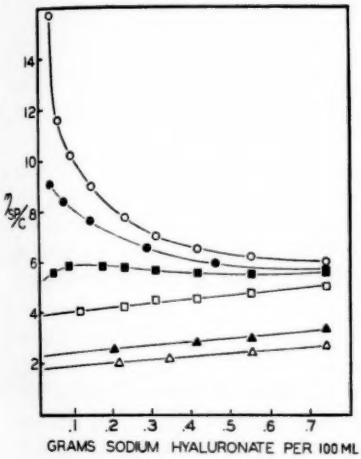

FIG. 3

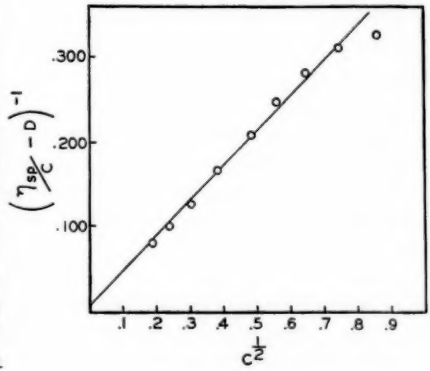

FIG. 4

FIG. 3. The reduced viscosity of sodium hyaluronate from the vitreous humor plotted against concentration. Concentrations of added sodium chloride, 1 M (△), 0.1 M (▲), 0.01 M (□), 0.003 M (■), 0.001 M (●), and without added salt (○).

FIG. 4. $(\eta_{sp}./c - D)^{-1}$ plotted against the square root of the concentration to test if Fuoss' equation is valid for hyaluronic acid. D equals 3 in the plot.

stiffer. The theory for polydisperse samples has been worked out only for the random coil model, and the results do not fit this function (10). It is therefore at present impossible to assign a shape to the vitreous hyaluronic acid molecule. However, if it was assumed that the particles were randomly kinked coils and that there was a large degree of polydispersity (z), the weight average molecular length ($z = \infty$ (10)) would be 3400 A.

The slope of the line Kc/R_0 *versus* c is identical with the second virial coefficient in van't Hoff's equation (14). It is slightly positive, indicating repulsive interaction between the particles.

Viscosity—The viscosity of Fraction B was measured in solutions of sodium chloride of various concentrations. Only a slight dependence of

the viscosity on the velocity gradient was found within the range 70 to 1400 sec.$^{-1}$, and the extrapolation to zero velocity gradient was easily done.

The intrinsic viscosity at ionic strength 0.1 is 2.3. If it is assumed that the particles are ellipsoidal, the axial ratio can be calculated according to Simha (16). From the intrinsic viscosity and the partial specific volume[3] (0.69) the viscosity increment should give an axial ratio of 70, assuming no hydration (17). Hyaluronic acid is, however, probably highly hydrated and the axial ratio is then considerably lower.

The viscosity at different ionic strengths as a function of the concentration follows the usual pattern for polyelectrolytes (18, 19), including hyaluronic acid from umbilical cord (20, 21) (Fig. 3). The upper curve representing sodium hyaluronate diluted with distilled water follows within the limits of experimental error Fuoss' well known equation (22)

$$\frac{\eta_{sp.}}{c} = \frac{A}{1 + B(c)^{\frac{1}{2}}} + D$$

where $\eta_{sp.}$ is the specific viscosity, c is the concentration in gm. per 100 ml., and A, B, and D are empirical constants (Fig. 4). However, the value of the intrinsic viscosity cannot be extrapolated within reasonable limits and is thus not useful for any calculations.

DISCUSSION

The method of preparation for hyaluronic acid described in this paper is based upon the fact that a large portion of the proteins of the vitreous body will precipitate together with some hyaluronic acid at pH 3 to 4. Even more proteins precipitate at low ionic strength. After deionizing the solution, the hyaluronic acid itself gives the required pH. The precipitate, which contains about 85 per cent of the total protein content and 35 per cent of the hyaluronic acid, is easily centrifuged. Most of the proteins still left in the supernatant solution can be precipitated together with some hyaluronic acid by increasing the ionic strength through saturation with sodium chloride. The hyaluronic acid then left in the supernatant liquid is fairly pure as judged from the analytical values, but may contain small amounts of proteins (5 per cent) and a small amount of galactosamine. The galactosamine content indicates contamination with another carbohydrate component.

The yield of the preparation is only about 60 per cent, since roughly half of the hyaluronic acid is precipitated with the proteins. The latter fraction can be recovered by removing the proteins by some other method (2, 3). It should be pointed out that the two fractions may very well have different molecular characteristics.

[3] Personal communication by Dr. L. Varga.

Blix has shown (23) that the first peak in the electrophoretic pattern of vitreous humor represents pure hyaluronic acid. The fact that it has been possible to prepare hyaluronic acid from the vitreous body without any drastic procedure confirms the concept that there is hyaluronic acid in the vitreous body which is not bound to proteins.

There is still a 5 per cent impurity of proteins in the preparation and an unknown carbohydrate component. If the impurities are species of higher molecular weight than hyaluronic acid, they will give erroneous results in the light scattering measurements. Apparently there is a high molecular weight impurity left after filtration which increases the molecular weight by 200 per cent. This impurity is removed by high speed centrifugation for 12 hours. 30 per cent of the protein nitrogen is centrifuged at the same time. The protein thus removed might well be a protein of the collagen class. The vitreous body contains vitrosin, a collagen type protein (24), and a certain amount of it could be dissolved at acid pH during the preparation. Light scattering data of collagens (25) suggest molecular weights between 4 and 24 $\times$ 10^6, and a contamination of 3 per cent with a substance of this molecular weight should increase the weight average molecular weight of the hyaluronic acid by the amount actually found.

The remaining impurity (about 3 per cent) still makes the results uncertain, as the scattering envelope seems to come from a multicomponent system. However, there are no indications that there are other giant molecules in the vitreous body with a molecular weight much larger than the one obtained, and this favors the theory of polydispersity as an explanation for the scattering envelope. Polydispersity of the hyaluronic acid from the vitreous body has been reported earlier, too (2, 26).[4]

The obtained molecular weight 340,000 to 500,000 is subject to a certain error (about 20 per cent) which has been discussed earlier (1). As it is a weight average molecular weight, the value tends to be too high, especially if the sample is highly polydisperse. Our value is in fairly good agreement with those reported by Brunish and Rowen, 500,000 (26), and Blix and Snellman, <250,000 (2).

There are repulsive forces among the molecules as indicated by the positive slope of Kc/R_0 *versus* c. This is contrary to the results obtained with hyaluronic acid from umbilical cord (1). However, in the latter case, the working concentrations were much smaller and it is possible that there is a small positive slope even with that preparation.

The viscosity values for sodium hyaluronate from the vitreous body are much lower than those from umbilical cord hyaluronate (1), as observed by many other workers (2, 3). The intrinsic viscosity in 0.1 M NaCl is only 2.3 and should, for an unhydrated ellipsoid, give an axial ratio of 70. The

[4] And personal communication.

true value is probably lower because of hydration. The value is consistent with a rod-shaped molecule built as a chain of disaccharides with a molecular weight of about 30,000. If the molecular weight is higher, however, as indicated by the light scattering measurements, another shape must be assigned, probably a random coil model. The large increase in the intrinsic viscosity, and thus in the size of the hydrodynamic unit at low ionic strength, may come from an increase of the length of the molecule (18, 19) or most probably from hydration (27).

SUMMARY

A mild method of preparation of sodium hyaluronate from the vitreous body, yielding a 90 per cent pure preparation, has been described. Light scattering reveals it as a multicomponent (or polydisperse) system with a weight average molecular weight of 340,000 to 500,000. The concentration dependence of the viscosity is typical of a polyelectrolyte. The intrinsic viscosity at ionic strength 0.1 is too low to be consistent with a stiff rod model for the molecule.

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AN ISOTOPE TRACER STUDY OF GLUCOSE CATABOLISM IN PSEUDOMONAS FLUORESCENS*

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The pseudomonads are characterized by a distinctive pattern of glucose catabolism in which the Embden-Meyerhof process apparently plays a minor rôle. From studies of Doudoroff and coworkers (1, 2) and others in various laboratories (3–11), there has emerged a new pathway, thus far exclusive with these organisms, involving the following successive transformations: glucose-6-phosphate → 6-phosphogluconate → 2-keto-6-phosphogluconate → 2-keto-3-deoxy-6-phosphogluconate → pyruvate + glyceraldehyde-3-phosphate → pyruvate. The finding of Entner and Doudoroff that arsenite-poisoned cells of *Pseudomonas saccharophila* yield 2 molecules of pyruvate labeled exclusively in the carboxyl carbon from glucose-1-C^{14} suggests that this organism utilizes this pathway exclusively. Further evidence of the occurrence of this or a similar pathway in an anaerobic, alcohol-producing species, *Pseudomonas lindneri*, has been reported by Gibbs and DeMoss (12) and DeMoss (13). The wide-spread formation of gluconic and 2-ketogluconic acids from glucose (3, 4, 8) indicates that this process occurs generally in the genus; however, it is not yet certain whether and to what extent other processes participate. Campbell and Norris (14) were unable to find any phosphorylated hexoses, and Wood and Schwerdt (8, 10) could not find kinases for phosphorylation of glucose or glucose-6-phosphate in *Pseudomonas fluorescens*. These observations argue against the occurrence of the Embden-Meyerhof process. On the other hand, the latter authors did find all of the other enzymes of the Embden-Meyerhof process, and Claridge and Werkman (15, 16) reported that sonic extracts of *Pseudomonas aeruginosa* phosphorylated glucose and gluconate in the presence of adenosine triphosphate. The occurrence of the direct oxidative pathway via pentose (hexose monophosphate shunt) in at least certain species is indicated by the ability of

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preparations from *P. fluorescens* to utilize ribulose- and ribose-5-phosphate, to produce small amounts of pentose phosphate from 6-phosphogluconate, and to form sedoheptulose from pentose (10).

The isotope data of Entner and Doudoroff (1) clearly indicate that the process discovered by them in *P. saccharophila* is essentially the only glucose catabolism route occurring in this organism under the conditions employed. The present study was undertaken to estimate to what extent this and other processes of glucose catabolism occur in intact cells of a common representative of this genus, *P. fluorescens*. An isotopic tracer procedure was employed for this purpose, variously C^{14}-labeled glucoses being utilized. As pointed out previously (17), data on specific activities of isolated intermediates derived from variously labeled glucoses allow the estimation of catabolism pathways, provided corrections are made for the endogenous metabolism. Data required for this correction are obtained by conducting simultaneous experiments with uniformly labeled glucose, as previously described (17). In the present report, such experiments are reported for *P. fluorescens*, NRRL B-6, based on the distribution and quantity of C^{14} present in acetate and pyruvate isolated after catabolism of glucose-1-, -2-, and -6-C^{14}.

EXPERIMENTAL

The organism used was a strain of *P. fluorescens* NRRL B-6, obtained from the Northern Regional Research Laboratory of the United States Department of Agriculture through the kindness of F. H. Stodola. Uniformly labeled glucose (glucose-U-C^{14}) was purchased from the Nuclear Instrument and Chemical Corporation of Chicago, and the glucose-1-, -2-, and -6-C^{14} were obtained through the kindness of Dr. H. S. Isbell from the National Bureau of Standards. These sugars were diluted with non-isotopic glucose to give specific activities of approximately 1000 c.p.m. when counted by our standard procedure. This involves conversion to barium carbonate, and measurement with a thin window tube of an "infinitely" thick layer 7.5 sq. cm. in area.

The cells were grown on either a 2 per cent glucose-salts (18) or a 2 per cent Difco Casitone-glucose medium. 500 ml. of medium (in 100 ml. aliquots) yielded 1 to 2 gm., dry weight, of cells when incubated at room temperature on a rotary shaker for 18 hours. The cells were harvested by centrifugation, washed twice with buffer at pH 6.8, and resuspended in buffer; aliquots of this suspension were used for the experiments.

Acetate Formation from Glucose Carbon—In these experiments an aliquot of the cell suspension equivalent to approximately 350 mg., dry weight, of bacteria was added to 100 ml. of a 0.067 M phosphate buffer solution of pH 6.8 in a 250 ml. Erlenmeyer flask containing 2 mmoles of the appropri-

ately labeled glucose and 2 mmoles of non-isotopic sodium acetate. Since acetate does not ordinarily accumulate under the conditions employed, its addition at the start of the experiment constituted a "trap" for metabolic acetate. An exactly similar flask was set up containing 2 mmoles of glucose-U-C^{14}. To insure constancy of conditions in each flask, both were run simultaneously and treated in exactly the same manner throughout the experimental period. The flasks were shaken side by side at room temperature on a rotary shaker, while a stream of CO_2-free oxygen was drawn through the flask and into a glass bead tower containing NaOH for absorption of respiratory CO_2. After 3 hours, the cells were killed by the addition of 6 ml. of 18 N H_2SO_4 and were centrifuged off and washed with

TABLE I

Products of Labeled Glucose Dissimilation by Resting Cells of P. fluorescens NRRL B-6

	Glucose-U-C^{14}			Glucose-1-C^{14}			
		RSA	Per cent total activity		RSA	Per cent total activity	Corrected RSA
	mmoles			*mmoles*			*per cent*
Glucose, start	2.0	100	100	2.0	100	100	
Acetate "	2.0	0	0	2.0	0	0	
" end	0.71	37.4	4.4	0.68	1.8	0.2	4.8
Respiratory CO_2, 0–2 hrs.	5.47	59.6	27.2	5.31	86.5	38.2	145
Respiratory CO_2, 2–3 hrs.	3.87	67.8	21.8	3.51	71.6	22.4	106

water. The acetate remaining was isolated from the supernatant solution and washings by steam distillation, and was assayed for radioactivity. Under these conditions about half of the glucose and about two-thirds of the acetate disappear, and appreciable quantities of the carbon of uniformly labeled glucose appear in both CO_2 and acetate (Table I). It is thus evident that acetate is an important intermediate in this organism, since it is oxidized and is formed readily from glucose. In contrast, glucose-1-carbon, though incorporated readily into CO_2, is incorporated into acetate at a low rate. To obtain an idea of the quantitative extent of the incorporation of glucose-1-carbon in acetate, it is necessary to correct for the production of acetate from endogenous carbon of the cells. This may be done, as previously described (17), in the following manner. The relative specific activity (RSA) of the acetate derived from the uniformly labeled glucose was 37.4 per cent, indicating a dilution by endogenous carbon of 62.6 per cent. We can assume that the glucose-1-C^{14} was diluted to a similar ex-

tent, and hence can calculate a corrected relative specific activity which the acetate from glucose-1-C^{14} would have had if it were not diluted by endogenous carbon. This value (corrected RSA) in the experiment of Table I is 1.8/0.374 = 4.8 per cent.

In Diagram 1 there are shown the three pathways of glucose catabolism under consideration. The Embden-Meyerhof process results in a symmetrical cleavage of the hexose chain into two triose units, ultimately yielding 2 pyruvate molecules. Thus, any pyruvate (or 3-carbon compound derived directly therefrom) produced from glucose-1-C^{14} via the Embden-Meyerhof process would have a corrected RSA of 100 per cent. In the process of oxidative decarboxylation to acetate, 2 unlabeled carbons are lost; hence any acetate derived via the Embden-Meyerhof process from glucose-1-C^{14} would have a corrected RSA of 150 per cent. The pentose or Entner-Doudoroff process, as outlined in Diagram 1, would not yield labeled acetate from glucose-1-C^{14}. In the former, carbon 1 is lost as CO_2 in pentose formation, and in the latter it is lost on decarboxylation of pyruvate. The observed low corrected RSA thus clearly indicates that not more than 4.8 × 100/150 = 3 per cent of the acetate produced from glucose came via the Embden-Meyerhof process. Data on three other similar experiments with glucose-1-C^{14}, summarized in Table II, are of similar magnitude; the respective corrected RSA values of 5, 18, and 6 per cent indicate that 3, 12, and 4 per cent of the acetates arose from glucose via the Embden-Meyerhof process.

Comparisons of CO_2 production from glucose-U-C^{14} and glucose-1-C^{14}, though not conclusively indicative of catabolism pathways,[1] are also in accord with the more decisive data already presented, indicating a relatively minor participation of the Embden-Meyerhof process. In this process, glucose carbon 1 becomes the methyl carbon of acetate and hence would be slow to appear in CO_2. The relatively high RSA of the respiratory CO_2 obtained during the metabolism of glucose-1-C^{14} is characteristic of the pentose or Entner-Doudoroff process, in which glucose carbon 1 is preferentially converted to CO_2.

Acetate from Glucose Carbons 6 and 2—In Table II there are summarized results obtained in three experiments identical with the preceding, except for the use of glucose-6-C^{14}. According to Diagram 1, acetate arising from glucose labeled in this position via the pentose path should have an RSA of at least 150 per cent (and possibly up to 300 per cent, depending on the

[1] The ratio of specific activities of CO_2 derived from glucose carbons 1 and 6 has been used as a means of estimating the relative extents of the Embden-Meyerhof and direct oxidative processes in various cell types (19–21). As pointed out clearly by Agranoff *et al.* (21), this ratio does not allow a calculation of the actual, but only of the minimal extent of the shunt. The values thus obtained may be highly misleading under certain circumstances.

DIAGRAM 1. Three pathways of glucose dissimilation possible in *P. fluorescens*. The numbering throughout is that of the original glucose, starting at aldehyde carbon. Sugar formulas are all in the open chain form.

extent to which the diose unit derived from glucose carbons 2 and 3 may be converted to acetate). Acetate arising via the Entner-Doudoroff process should also have a specific activity of 150 per cent. However, the observed values ranged from 92 to 107 per cent. Though these results are thus somewhat in conflict with the preponderant occurrence of either one of these two mechanisms alone, they are in accord with the simultaneous operation of both mechanisms. In the pentose process, glucose-6-C^{14} would yield 3-labeled triose phosphate which, by the classical aldolase reaction, would yield 1,6-labeled hexose diphosphate. Assuming that cellular mechanisms exist for its conversion to hexose monophosphate, the subsequent operation of either the pentose or the Entner-Doudoroff process on this 1,6-labeled hexose would lead to the loss of C^{14} for acetate formation

TABLE II

Glucose Catabolism by P. fluorescens in Presence of "Acetate Trap"

Substrate	Corrected RSA, per cent	
	Acetate	Respiratory CO_2
Glucose-1-C^{14}	5.0	130
	18.0	130
	6.0	137
Glucose-6-C^{14}	107	80
	94	100
	92	94
Glucose-2-C^{14}	96*	96

* Distribution, methyl, 21 per cent; carboxyl, 79 per cent.

Data on incorporation of glucose-6-C^{14} into CO_2 are consistent with this idea. If glucose carbon 6 were exclusively converted to the methyl carbon of pyruvate, its conversion to CO_2 might be expected to be slower than that of the other carbons; however, the relative specific activities of 80 to 100 per cent observed in these experiments indicate that this carbon atom is converted to CO_2 about as rapidly as are the other glucose carbons.

More definitive evidence in favor of the operation of the Entner-Doudoroff and pentose processes was obtained in the experiment with glucose-2-C^{14} shown in Table II. The relatively high RSA, 96 per cent, indicates that this carbon contributes substantially to acetate formation. As shown in Diagram 1, acetate can arise directly from this position via all three processes, the Entner-Doudoroff and Embden-Meyerhof pathways yielding carboxyl-labeled, and the pentose pathway methyl-labeled, acetate. The acetate obtained had 79 per cent of its C^{14} in the carboxyl carbon. Since

glucose carbon 1 contributed only slightly to acetate, it can be assumed that the carboxyl-labeled acetate arises from carbons 2 and 3 of glucose via the Entner-Doudoroff process. On this basis it can be calculated that $96 \times 0.79 \times 100/150 = 50$ per cent of the acetates arose via the Entner-Doudoroff process. This estimate is maximal, since it does not take into account a small amount of acetate arising from carbons 1 and 2 via the Embden-Meyerhof process. Since we have seen that this process may account for up to 12 per cent of the acetates formed, it is possible that the estimate of 50 per cent may be high to this extent. The 21 per cent of methyl-labeled acetate conceivably may arise by a direct split of pentose into C_2 and C_3, followed by conversion of C_2 to acetate. Though no direct evidence of such a split is as yet available without the intervention of an acceptor in the transketolase reaction, it is conceivable that glycolaldehyde can be directly converted to acetate, since both compounds are at the same oxidation level. Isotopic evidence for such a conversion has been obtained by Gunsalus and Gibbs (22) in the fermentation of glucose by *Leuconostoc mesenteroides*, and other evidence for such a reaction in the catabolism of pentoses is available (23). Sprinson and Weliky (24) have recently shown that D-ribose-1-C^{14} and D-xylose-1-C^{14} are converted to acetate-2-C^{14} in the rat. As before, we can estimate that $96 \times 0.21 \times 100/150 = 13$ per cent of the acetate could have arisen by this mechanism.

However, it also is conceivable that the small quantity of methyl-labeled acetate may have arisen from glucose carbon 2 via a combination of the pentose and Entner-Doudoroff pathways. Diagram 2 lists two reaction pathways undergone by intermediates of the shunt in the transaldolase and transketolase reactions (25, 26), resulting in the reformation of hexose. In Reaction 1, carbon present originally in glucose carbon 2 would appear in glucose carbons 1 and 3; subsequent operation of the Embden-Meyerhof or Entner-Doudoroff process on this reformed hexose would yield methyl-labeled acetate. Similarly, the occurrence of Reaction 2 would lead to reformed hexose labeled in carbon 1, and this could yield a small quantity of methyl-labeled acetate via the Embden-Meyerhof pathway. With no means of quantitative evaluation of these reactions, the estimates based on results with glucose-2-C^{14} are not entirely certain. However, they are in agreement with data on glucose-1- and -6-C^{14} in indicating that in this organism the Entner-Doudoroff and pentose processes participate roughly equally in the catabolism of glucose to acetyl groups.

Formation of Pyruvate from Glucose-1- and Glucose-6-C^{14}—To bring about the accumulation of pyruvate in these experiments with *P. fluorescens*, the procedure described by Entner and Doudoroff (1), *viz.* adding sodium arsenite in a final concentration of 0.002 M to the buffered glucose solution, was

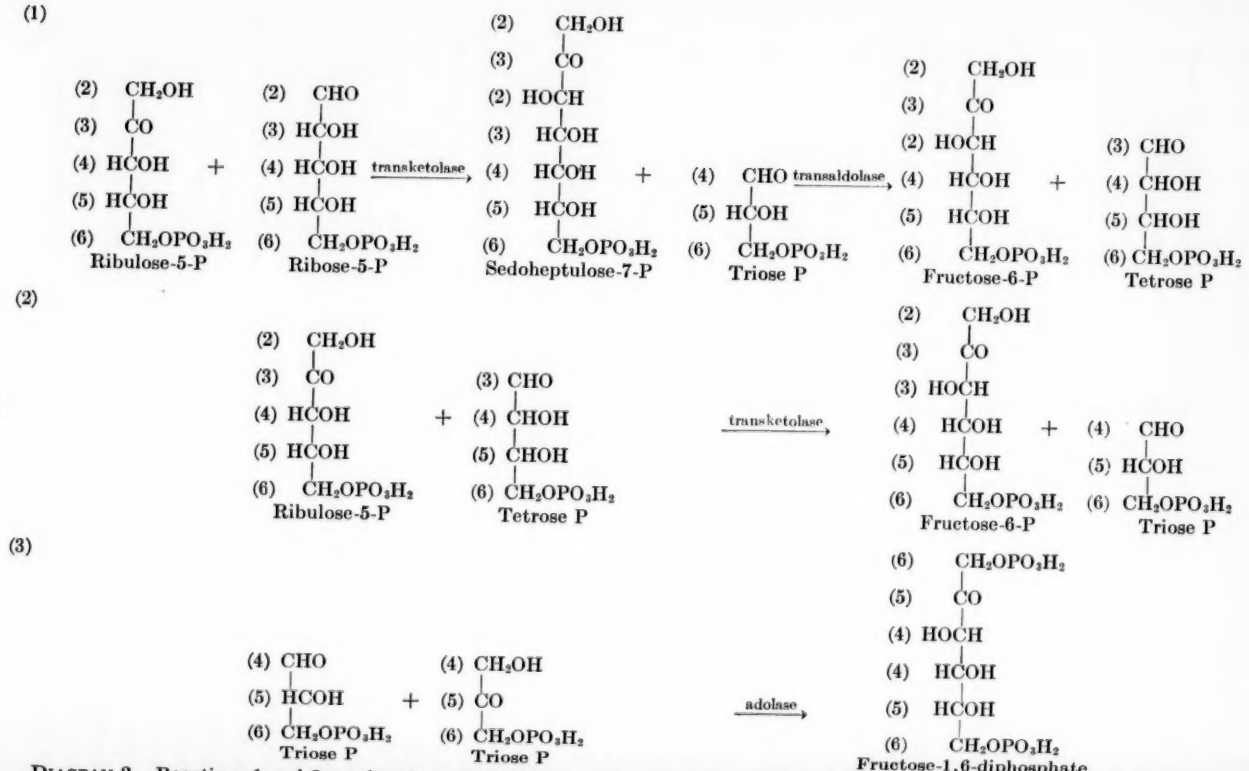

DIAGRAM 2. Reactions 1 and 2 catalyzed by transaldolase and transketolase; Reaction 3, by classical aldolase. The numbering of carbon atoms corresponds to those of the original glucose.

followed. Oxidation of glucose under these conditions was somewhat slower and, to obtain appreciable reaction, the incubation period was extended to 6 hours.

After centrifugation of the bacterial cells, an excess of 0.1 per cent 2,4-dinitrophenylhydrazine solution in 2 N HCl was added to the supernatant solution, and the mixture was allowed to stand overnight at room temperature. It was then extracted successively with ethyl ether until no more color was extracted. The ether solution was evaporated to a small volume, and the hydrazones extracted from the ether layer by shaking with 10 per cent sodium carbonate solution. The solution of the sodium salts was acidified with sulfuric acid, the 2,4-dinitrophenylhydrazones were reextracted with ether, and the ether solution was evaporated to dryness. The pyruvic acid hydrazone thus obtained was usually, though not always, accompanied by small quantities of the hydrazone of α-ketoglutaric acid.

In order to separate the mixture by paper chromatography, 50 mg. of the hydrazones were dissolved in 30 ml. of ethyl ether and the solution was spread in a horizontal line 1.5 inches from the bottom of fifteen 18 × 14 inch rectangular sheets of Whatman No. 3 filter paper. These were arranged in cylinders formed by stapling the edges together, and placed concentrically in covered glass jars, each holding 250 ml. of 0.05 N NaOH. The chromatograms were allowed to develop for 5 hours, and dried in air. Each sheet had three distinct, well separated yellow bands with R_F values of 0.67, 0.58, and 0.41. Chromatography of the pure hydrazones of α-ketoglutarate and pyruvate established that the upper band corresponded to α-ketoglutarate, and the two lower bands to pyruvate (27). We also consistently observed a brown streak at the origin and a purplish blue band with R_F 0.25, which was partially identified as the hydrazone of either glyceraldehyde or dihydroxyacetone. Other unidentified bands of various shades of blue, brown, and red also appeared in isolated experiments.

The pyruvate and α-ketoglutarate bands were cut from the sheets and the hydrazones stripped from the paper by descending chromatography with water as the eluent. The aqueous solution was acidified with dilute H_2SO_4, and the pure compounds isolated by extraction with ether and recrystallization from 50 per cent ethanol. The pyruvic acid 2,4-dinitrophenylhydrazone was converted to CO_2 and acetic acid by oxidation with acid permanganate, according to the method of Krebs (28). The acetate was further degraded by the Phares-Schmidt procedure (29).

The results of a representative experiment of this type are summarized in Table III. In contrast with similar experiments with *P. saccharophila* reported by Entner and Doudoroff (1), production of pyruvate was not quantitative; in fact, an amount of glucose carbon almost equivalent to the amount converted to pyruvate escaped the arsenite block and appeared

Triose 1 Triose 2 Fructose-1,6-diphosphate

DIAGRAM 2. Reactions 1 and 2 catalyzed by transaldolase and transketolase; Reaction 3, by classical aldolase. The numbering of carbon atoms corresponds to those of the original glucose.

XUM

as CO_2. The absence of a cosubstrate such as was employed in the acetate experiments was reflected in the relatively high relative specific activities of the pyruvate and of the CO_2. In this experiment, data on glucose-U-C^{14} indicate that only about 30 per cent of the pyruvate or the CO_2 arose from endogenous carbon.

The low corrected RSA of the pyruvate derived from glucose-1-C^{14} provides clear evidence that a process other than the Embden-Meyerhof or Entner-Doudoroff process is participating in a major fashion in glucose

TABLE III

Catabolism of Glucose by Resting Cells of P. fluorescens in Presence of Arsenite

Cells were suspended in 0.067 M phosphate buffer, pH 6.8, containing arsenite in 0.002 M concentration. Shaking was conducted for 6 hours.

Product isolated	Glucose-U-C^{14}			Glucose-1-C^{14}				Glucose-6-C^{14}			
		RSA	Per cent total activity		RSA	Per cent total activity	Corrected RSA, per cent		RSA	Per cent total activity	Corrected RSA, per cent
	mmoles			*mmoles*				*mmoles*			
Respiratory CO_2											
0–3 hrs.	1.24	67	6.9	1.24	137.5	14.2	205	1.27	33.5	3.4	50
3–6 "	1.47	78	9.6	1.52	131.0	16.5	168	1.50	27.8	3.4	36
Total.....	2.71		16.5	2.76		30.7		2.77		6.8	
Pyruvate	1.26	72.2	22.7	1.27	21.6	6.8	30	1.26	104	33.0	144
COOH						91				22	
CO						} 9				3	
CH_3										75	

catabolism in this organism. Pyruvate arising exclusively via these pathways from either glucose-1-C^{14} or glucose-6-C^{14} would have an RSA of 100. The observed RSA of 30 signifies that only about 30 per cent of the pyruvates from glucose-1-C^{14} could have arisen from these processes. Inasmuch as 91 per cent of the labeled pyruvate carbon was in the carboxyl group, one can estimate that $0.91 \times 30 = 27$ per cent of the pyruvates arose via the Entner-Doudoroff process and $0.09 \times 30 = 3$ per cent arose via the Embden-Meyerhof pathway. These data are in only rough quantitative agreement with the acetate experiments, but they are in good accord with these in indicating (*a*) that the Embden-Meyerhof pathway is not quantitatively significant, (*b*) that the Entner-Doudoroff process is a major path-

way, and (*c*) that a process such as the pentose path, in which pyruvate is derived solely from glucose carbons 4 to 6, is also of major quantitative significance.

Data on pyruvate from glucose-6-C^{14} are in agreement with this concept. If all of the pyruvate arose via the Entner-Doudoroff process, the RSA from glucose-6-C^{14} could not have been greater than 100. The fact that it is 144 indicates again that there is a preferential utilization of carbons 4 to 6 for pyruvate formation, and again points to a process such as the pentose in which carbon 6 is preferentially utilized for pyruvate formation as a major process. This RSA of 144 was somewhat lower than may have been anticipated on the basis of the data from glucose-1-C^{14}. If the Entner-Doudoroff process occurred exclusively, the RSA would have been 100. If the pentose process occurred exclusively, the RSA would have been 200. On the basis of 30 per cent Entner-Doudoroff calculated on the basis of the glucose-1-C^{14} data, the RSA should have been 170. The difference from the observed value of 144 can probably reasonably be attributed to the explanation already invoked to explain the low RSA of acetate from glucose-6-C^{14}, *viz.* the migration of glucose-6-carbon to carbon 1 via Reaction 3 of Diagram 2. The glucose-1-C^{14} thus produced should lead to the loss of an appreciable portion of the labeled carbon through further breakdown of the glucose via the oxidative process. Part of this newly formed glucose-1-C^{14} should undergo catabolism via the Entner-Doudoroff process, and this would result in the formation of carboxyl-labeled pyruvate. In accord with this expectation, 22 per cent of the C^{14} of pyruvate derived from glucose-6-C^{14} was in the carboxyl group. It is possible, of course, that the partial randomization of pyruvate labeling may have been due to the formation of symmetrical triose such as dihydroxyacetone. As yet, however, such an occurrence is uncertain.

Data on CO_2 production in the pyruvate experiments are also in accord with a preferential conversion of glucose carbon 1 to CO_2. As seen in Table III, the RSA of CO_2 from glucose-1-C^{14} was about 4 times higher than that from glucose-6-C^{14}. Indeed, in all experiments the specific activity of respiratory CO_2 derived from glucose-1-C^{14} rose more rapidly, reached a higher maximum, and declined more rapidly than did that from glucose-U-C^{14} or glucose-6-C^{14}.

DISCUSSION

The quantitative data calculated from the results of these experiments are not to be regarded as exact estimations. The calculations are based on a direct conversion of glucose to C_2 or C_3 units via the routes of Diagram 1. It is already evident that triose formation via the oxidative process may proceed by devious routes, as in Diagram 2, which scatter the

labeled carbon throughout the various products. The possibility also exists that labeled carbon might enter C_2 or C_3 compounds via CO_2 fixation or other as yet unknown pathways, and the further possibility must be considered that triose phosphates may not come into equilibrium. Added to these uncertainties is the possibility that the addition of an inhibitor such as arsenite to cause the accumulation of pyruvic acid might change the pattern of glucose catabolism. Since our results are reasonably self-consistent, with no anomalous results to contend with, we feel that they represent reliable approximations, based on our present knowledge of glucose catabolism.

With these qualifications we conclude that resting cells of this organism use both the Entner-Doudoroff and pentose pathways as major routes of glucose catabolism. Probably one-third to one-half of the glucose is metabolized via the Entner-Doudoroff process. Most of the remainder is catabolized by a mechanism which does not involve the formation of either acetate or pyruvate from glucose-1-carbon but does involve their formation from glucose-6-carbon. In all likelihood this mechanism is the direct oxidative path; this path is consistent with the preferential conversion of glucose-1-carbon to CO_2, and no other pathway is known which would account for these observations. It is not yet certain what happens to the diose unit arising from carbons 2 and 3. The data on acetate formation from glucose-2-C^{14} indicate that relatively little is directly converted to acetate; probably much undergoes recycling via the pathways shown in Diagram 2, yielding new hexose molecules which may be broken down in turn via either of the two processes.

The results of this study clearly eliminate the Embden-Meyerhof process as a quantitatively significant mechanism of glucose catabolism in this organism, which was replaced in part by a process which yields the same end-result; *viz.*, the formation of 2 molecules of pyruvate from hexose by a wholly different path. This pattern of glucose catabolism is not restricted to the strain of *P. fluorescens* used in the reported studies, since essentially similar quantitative results attributable to the same general catabolic pattern were obtained with a strain of *P. aeruginosa* obtained from W. L. Gaby and with sucrose-grown *P. saccharophila* obtained through the kindness of M. Doudoroff. Though the pseudomonads appear to be unique in this respect, it is conceivable that further study will reveal other organisms which utilize this means of glucose catabolism.

SUMMARY

Acetate produced by glucose-grown, resting cells of *Pseudomonas fluorescens* oxidizing uniformly C^{14}-labeled glucose had a high relative specific

activity (RSA), indicating that acetate is a major intermediary metabolite in this organism. Acetate produced from glucose-1-C^{14} had a low RSA, whereas that from glucose-6-C^{14} had a high RSA. Glucose-2-C^{14} yielded acetate with a high RSA, labeled 79 per cent in the carboxyl and 21 per cent in the methyl carbon. Pyruvate, obtained in similar experiments with arsenite-poisoned cells, had a low RSA when derived from glucose-1-C^{14}, with 91 per cent of its activity in the carboxyl carbon. Pyruvate from glucose-6-C^{14} had a high RSA from glucose-6-C^{14} with 75 per cent of its activity in the methyl group. CO_2 production from glucose carbon 1 was always higher than, and from glucose carbon 6 always lower than, that from the average of all carbons (obtained from data on uniformly labeled glucose). Calculations from these data provide evidence for the following assumptions: (*a*) the Embden-Meyerhof process occurs to a very small extent; (*b*) the pentose process is a major one, accounting for probably one-half or more of the glucose molecules catabolized; (*c*) the Entner-Doudoroff process also occurs to a major extent, approximately one-third to one-half of the glucose molecules being catabolized thereby.

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DEOXYRIBOSIDES IN ANIMAL TISSUES*

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Although cytological and cytochemical studies have demonstrated that deoxyribose nucleic acid (DNA) is localized exclusively in the nucleus of most cells, the existence of precursors of DNA as normal constituents of cells has not been satisfactorily established. Compounds with deoxyribosidic properties have been detected microbiologically in liver and bacterial extracts (2, 3) and in frog eggs (4), and, more recently, thymidylic acid has been reported to have been isolated from the acid-soluble fraction of liver (5). It is not clear from these reports, however, whether the compounds detected were normal constituents of the cells studied or autolytic products. With respect to this question, Dorough and Seaton (6) reported that thymidine and thymidylic acid were not present in extracts of most fresh or aged normal mouse tissues. Since these workers were unable to recover completely compounds, such as cytidine, added to tissues, their failure to detect thymidine and thymidylic acid in tissue extracts may not be significant.

In order to explore this problem more fully, tissues were obtained from animals under conditions known to correspond closely to those *in situ* (*i.e.*, from anesthetized animals frozen in liquid nitrogen (7)) and acid extracts were prepared. Assay of these extracts with the organism *Lactobacillus acidophilus* R-26 (8) revealed that small but significant amounts of deoxyribosidic compounds were present in a wide variety of normal and tumor tissues as well as in the blood of rats and mice and evidence has been obtained indicating that deoxycytidine was present in rat blood and that deoxycytidine and deoxyuridine were present in rat liver.

Materials and Methods

The following compounds were used in this study: thymidine, m.p. 185–186°; deoxyadenosine, m.p. 191–192°; deoxyguanosine; deoxyuridine, m.p. 159–160°; deoxycytidine hydrochloride, m.p. 164–165°; calcium thymidylate (dihydrate); diammonium deoxycytidylate (dihydrate); deoxyguanylic acid; ammonium deoxyadenylate (trihydrate).[1] Other chemicals

* A preliminary report of some of the material reported in this paper has appeared (1).

[1] With the exception of thymidine, all of the deoxy compounds were products of

used were reagent grade commercial products. Enzyme-hydrolyzed casein was a product of General Biochemicals, Inc.

Preparation of Tissue Extracts—Unless otherwise specified, tissues[2] either were obtained as rapidly as possible from animals under ether anesthesia and plunged into liquid nitrogen or were dissected from anesthetized animals that had been frozen in liquid nitrogen (7). In most cases the tissues from several animals were pooled to obtain sufficient material. Blood was obtained from etherized animals by severing the neck vessels and bleeding directly into liquid nitrogen. Blood plasma was prepared from heparinized blood obtained without freezing. The blood and tissues were powdered, weighed, and homogenized as rapidly as possible with a volume of ice-cold 12 per cent trichloroacetic acid (TCA)[3] equivalent to twice the weight of the tissue. The resulting suspensions were centrifuged for 20 to 30 minutes at 0–2° and 17,500 r.p.m. in the International model PR-1 centrifuge (rotor No. 296). The supernatant fluids were removed and extracted four times with 1 volume of ether before neutralizing to pH 7 to 7.4 with dilute NH_4OH. Dissolved ether was removed under reduced pressure. The neutralized extracts were used as such for the estimation of total deoxyriboside content.

For chromatography, concentrates of the tissue extracts were made as follows: 9 volumes of absolute ethanol were added to the neutral liver extracts and the mixture was kept at 0° for 45 to 60 minutes before centrifuging precipitated materials. The supernatant fluid was evaporated to dryness under reduced pressure (Rinco rotating evaporator, A. S. Aloe Company) and the residue was redissolved in water and made up to one-tenth the original extract volume. Neutralized blood extracts were evaporated to dryness directly, redissolved in water to one-tenth the original volume, and then precipitated with 9 volumes of ethanol. After centrifuging, the supernatant fluid was evaporated to dryness and the residue was redissolved in water to one-tenth the original volume. Essentially none of the deoxyriboside content of the extracts was lost during concentration. In five experiments with liver extracts, an average of 98 per cent (90 to 104) of the original deoxyriboside content was recovered in the concentrated alcohol-soluble fraction. Similar recoveries have also been obtained with blood and other tissues. The ethanol precipitation removed approximately

the California Foundation for Biochemical Research. These products were chromatographically homogeneous and 100 ± 3 per cent pure on the basis of spectrophotometric assay. Two samples of thymidine were used. One was a product of the Schwarz Laboratories (lot No. TDN 5304). The other was a gift from Dr. E. E. Snell.

[2] The author is indebted to Dr. E. Shelton, Dr. R. E. Greenfield, and Dr. A. J. Dalton for supplying the tumors used in this study.

[3] Identical results have been obtained with perchloric acid extracts.

80 per cent of the material absorbing at 260 mμ and 45 per cent of the total nitrogen content of the extracts.

Paper Chromatography—0.05 to 0.2 ml. aliquots of the ethanol-soluble concentrates were applied in small portions (0.6 × 2.7 cm. spots) 8 cm. from one end of 3.8 × 61 cm. strips of Whatman No. 1 paper. In large scale separations, Whatman No. 3 MM paper provided with a Whatman No. 1 wick was used and approximately 1.6 ml. of tissue concentrate were applied to each strip. In each experiment, 40 γ samples of the pure deoxyribosides were run simultaneously on separate strips. Descending chromatography was employed with *n*-butanol saturated with water (NH_3 atmosphere (9)), *n*-butanol saturated with 5 per cent aqueous boric acid (10), or water-saturated *n*-butanol containing acetic acid (5 ml. of acetic acid in 25 ml. of butanol-water (11)). The chromatograms were developed for 22 to 24 hours, during which time the solvent traveled 45 to 50 cm. beyond the point of application of the sample.

The effect of chromatographing the pure pyrimidine deoxyribosides in the presence of 0.1 ml. of a liver concentrate was determined and it was found that the separation of these compounds was affected only slightly. The average position of the nucleosides was displaced slightly by the liver extract and the bands were somewhat broader than in the absence of liver extract.

Determination of Deoxyribosides—The deoxyriboside content of the tissue extracts was estimated microbiologically with the organism *L. acidophilus* R-26 (ATCC 11506) maintained as described by Hoff-Jørgensen (8). The assay medium used was the same as that specified by him, except that commercial enzyme-hydrolyzed casein was used at double the concentration. The turbidity resulting from the growth of the organism was measured at 660 mμ with 1 cm. cuvettes in the Beckman model DU spectrophotometer equipped with a photomultiplier attachment. With pure solutions of thymidine, the turbidities produced were directly proportional to concentration in the range 0.04 to 0.16 γ per ml. Assays of tissue extracts were made by selecting four to six aliquots producing growth responses in this range and averaging the results.

In determining the distribution of tissue deoxyribosides on paper chromatograms, the paper was dried in the hood and cut into transverse strips 0.5 to 1.0 cm. wide. Each strip was autoclaved with 1.5 ml. of water (0.01 N NH_4OH in the case of boric acid chromatograms) and 1.5 ml. of double strength medium (8). The tubes were then inoculated and incubated for 18 to 24 hours at 37° and the turbidities were measured. The small amounts of fibers introduced into the medium from the paper strips produced negligible turbidities.

Other Determinations—Tests for deoxyribose on paper chromatograms

were made with the cysteine-sulfuric acid reagent (Buchanan (12)). The cysteine-sulfuric acid tests on eluates of paper chromatograms were made by the method of Dische (13) as modified by Stumpf (14) with deoxycytidine and deoxyuridine as standards. Paper chromatograms were examined under ultraviolet light at 260 mμ (SL Mineralight SV-41) to ascertain the presence of purine- and pyrimidine-containing compounds as well as to locate the positions of the pure deoxyribosides on control chromatograms. Ultraviolet spectra of eluates were determined in the Beckman spectrophotometer.

Results

Hoff-Jørgensen (8) reported that *L. acidophilus* R-26 specifically requires deoxyribosides for growth and showed, indirectly, that deoxyribotides were as effective in inducing growth as deoxynucleosides. DNA was inactive. We have repeated and confirmed most of Hoff-Jørgensen's tests and, in addition, have studied the pure deoxynucleotides and found them to be as active as the nucleosides in promoting growth.

Tissue Distribution of Deoxyribosidic Compounds—Microbiological assay of extracts of a wide variety of rodent tissues showed that deoxyribosides or deoxynucleotides were present in small amounts in all of them (Table I). The concentration of these compounds in all normal mouse tissues, with the exception of spleen, was very low compared with that in normal rat tissues. The fact that the content of these compounds in tissues such as liver did not vary significantly whether the tissue was removed from frozen anesthetized animals, from animals under anesthesia, from decapitated animals, or from animals a few minutes post mortem would seem to indicate that deoxyribosides exist normally in tissues *in situ*. The total content of deoxyribosidic compounds in all mouse tumors studied was considerably higher than that in normal mouse tissues (except spleen), but the concentration in the only rat tumor analyzed did not differ significantly from that in normal rat liver.

Nucleoside Nature of Growth-Promoting Factors—The behavior of the growth-promoting factors present in tissue extracts on ion exchange columns and paper chromatograms provided evidence for their nucleoside nature. As shown by the data in Table II, almost the entire growth-promoting activity of liver and blood extracts passed through Dowex 1 columns used under conditions favorable to the retention of nucleotides (15). On the other hand, almost the entire growth-promoting activity of the liver eluate from Dowex 1 was retained on a Dowex 50 column. The latter behavior is typical of some deoxyribosides, although not a unique property of these compounds. When the tissue extracts were chromatographed on paper with the solvents described above, most of the growth-promoting

activity migrated considerable distances from the starting point. Under these conditions of chromatography, none of the deoxynucleotides migrates appreciably.

TABLE I

Deoxyriboside Content of Animal Tissues

Species	Strain	Tissue	Deoxyriboside content*
			γ per gm. fresh tissue
Mouse	C3H	Liver	0.81,† 0.81, 0.80, 0.66, 0.80
	dba	"	0.58
	A	"	0.88
	C3H	Brain	0.67
	"	Kidney	1.23
	"	Lung	1.31
	"	Spleen	15.2, 9.7
	"	Blood	1.0, 0.83,‡ 0.93‡
	"	Hepatoma 37	2.5, 5.4, 3.1, 3.8
	"	" 98/15	5.1, 5.0
	dba	Thymoma	42.6, 22.6
	C3H	Mammary carcinoma	5.7
	A	Lymphoma 1 cells	24.4, 26.9
	"	" 1 ascitic fluid	11.5, 13.4
		Sarcoma 37	2.6
Rat	Holtzman	Liver	14.8, 12.7, 14.3,§ 12.4, 12.1,‖ 11.2, 11.4,† 12.8, 11.5,§ 16.5
	"	Lung	8.7
	"	Brain	3.2
	"	Spleen	17.3, 30.7
	"	Kidney	12.1, 20.9
	"	Blood	13.6, 10.6,¶ 7.5,¶ 10.4, 12.7
	"	" plasma	14.1
	"	Novikoff hepatoma	12.3, 10.1, 14.9

* Expressed as thymidine.
† Animals decapitated without anesthesia.
‡ Hepatoma 37-bearing animals.
§ Animals frozen in liquid air.
‖ Removed 2 minutes after death of animal.
¶ Hepatoma-bearing animals.

Absence of Purine Deoxynucleosides—Since the above results indicated that deoxyribosides were the principal compounds being measured, it became important to determine which deoxyribosides were present. As demonstrated in Table III, the ability of deoxyadenosine to promote growth was completely lost after heating at pH 2, while the activity of thymidine

was retained. The difference in behavior of these two compounds toward hydrolysis at pH 2 is typical of the difference in behavior of purine deoxyribosides and pyrimidine deoxyribosides in general (*cf.* (8)). Since the deoxyribosides present in tissue extracts were resistant to hydrolysis at

TABLE II

Behavior of Rat Liver and Blood Extracts on Ion Exchange Columns

Neutral liver or blood extracts were passed through a 0.9 × 4 cm. Dowex 1 formate column (15) and the column was washed with water. One liver eluate was also passed through a 0.9 × 3 cm. Dowex 50 (H^+ form) column (15).

Material	Deoxyriboside content*
Liver extract	11.2
Eluate from Dowex 1	9.2
" " " 50	0
Blood extract	13.6
Eluate from Dowex 1	14.1

* Expressed as micrograms of thymidine per gm. of liver or blood.

TABLE III

Behavior of Adenine and Thymine Deoxyribosides and of Growth-Promoting Activity of Extracts Toward Hydrolysis at pH 2

Solutions of the pure deoxyribosides (1.0 γ per ml.) or of the tissue extracts were acidified to pH 2 with dilute HCl and heated for 5 minutes in boiling water. After cooling and neutralizing, the solutions were tested for growth-promoting activity.

Material	Deoxyriboside content*
Deoxyadenosine	0
Thymidine	92
Rat liver extract	94
Thymoma extract	89
Rat hepatoma concentrate	95
Blood concentrate (hepatoma-bearing rats)	104

* Expressed as per cent of unheated controls.

pH 2 (Table III), it can be concluded that purine deoxyribosides were absent and that the active compounds were pyrimidine deoxyribosides.

In order to test the possibility that purine deoxyribosides were lost during the preparation of the tissue extracts owing to the acid lability of these compounds, 100 γ of deoxyadenosine were added to 10 ml. of 12 per cent TCA. After 1 hour at 0°, the TCA was removed with ether as usual. Microbiological assay of the residue showed that 91 per cent of the added

nucleoside remained. In another experiment a 50 per cent rat liver homogenate in water was rehomogenized with an equal volume of 18 per cent TCA containing 180 γ of deoxyadenosine per ml. After removing the protein by centrifugation, the concentration of deoxyadenosine in the supernatant solution was found to be 88 per cent of that in the deoxyadenosine-containing TCA diluted with an equal volume of water. The absence of purine deoxyribosides in tissue extracts could thus not be explained on the basis of decomposition or adsorption.

Paper Chromatography of Tissue Deoxyribosides—The positions of the peaks of growth-promoting activity on the paper chromatograms of liver and blood also indicated that these compounds were pyrimidine deoxyribosides. The average R_F values of the bands of growth-promoting activity (Table IV) corresponded closely with those of pure deoxycytidine, deoxyuridine, or thymidine run simultaneously. Although the R_F values of the tissue deoxyribosides varied considerably in different experiments, those of the pure deoxyribosides varied in a parallel manner.

In the case of rat blood, a homogeneous chromatographic pattern was observed (Table IV). The main component in butanol-water and in butanol-boric acid corresponded to deoxycytidine. In addition, smaller amounts of a deoxyuridine component were observed in some of the butanol-boric acid chromatograms. The presence of a deoxyuridine component in the latter chromatograms was compatible with the behavior of the pure deoxyribosides in the two solvent systems, since butanol-water did not adequately resolve deoxycytidine and deoxyuridine.

In the paper chromatography of the rat liver deoxyribosides, consistent results were obtained with butanol-water, while with butanol-boric acid the results were conflicting with different samples although readily reproducible with a given sample. The data obtained from chromatographing eleven different liver concentrates (Table IV) are grouped, for the sake of brevity, into one of three classes depending upon the solvent used and whether one, two, or three deoxyriboside components were observed. Chromatography of the first sample of rat liver concentrate prepared revealed a single band corresponding to deoxycytidine in butanol-water and two bands corresponding to deoxycytidine and deoxyuridine in butanol-boric acid. These results were considered to be confirmatory, since the deoxyribosides were more adequately resolved in butanol-boric acid. Chromatography of three additional liver samples in this solvent also revealed deoxycytidine and deoxyuridine components. At this point, an entirely different pattern was observed in butanol-boric acid chromatograms of liver. In seven samples tested, the main component was a rapidly migrating one corresponding to thymidine. Smaller amounts of deoxycytidine and deoxyuridine were also present. In view of this greatly altered

TABLE IV

Paper Chromatography of Rat Liver and Blood Extracts

The main components are given in bold-faced type. The R_F values represent the ratios of the average distance traveled by the spot to that traveled by the solvent front. The figures in parentheses represent the range of values observed.

Tissue	No. of experiments	Deoxyriboside content	Recovery on chromatogram	Growth zones			Deoxyriboside*			
				1	2	3	Cytosine	Uracil	Thymine	Solvent†
		γ per gm.	per cent	R_F	R_F	R_F				
Blood	2	11.6	40	**0.22**			0.22	0.27	0.44	
		(10.4–12.7)	(29–50)	(0.19–0.24)			(0.20–0.24)	(0.25–0.28)	(0.41–0.46)	1
"	4	13.0	63	**0.31**	0.49‡		0.32	0.48	0.62	
		(10.4–15.1)	(54–70)	(0.27–0.35)	(0.47–0.50)		(0.26–0.39)	(0.44–0.52)	(0.55–0.67)	2
Liver	6	12.6	44	**0.24**			0.24	0.26	0.46	
		(11.2–16.3)	(33–65)	(0.21–0.26)			(0.22–0.27)	(0.24–0.28)	(0.44–0.48)	1
"	4	13.6	78	**0.29**	**0.40**		0.29	0.40	0.56	
		(10.2–19.3)	(69–85)	(0.24–0.35)	(0.39–0.40)		(0.24–0.34)	(0.39–0.40)	(0.51–0.61)	2
"	7	12.5	86	0.24	0.37§	**0.53**	0.26	0.40	0.55	
		(11.2–16.3)	(72–98)	(0.23–0.27)	(0.34–0.39)	(0.49–0.56)	(0.24–0.28)	(0.36–0.42)	(0.52–0.57)	2

* Deoxyguanosine and deoxyadenosine were also run in these experiments. The former compound had the lowest R_F while the R_F of the latter was intermediate between that of thymidine and deoxyuridine.

† Solvent 1, *n*-butanol saturated with H_2O; Solvent 2, *n*-butanol saturated with 5 per cent boric acid.

‡ This component was present in only two of the four experiments.

§ Deoxyribosidic activity was present in this zone in all experiments, but evidence for a discrete band was present in only four experiments.

chromatographic pattern, the last five of these samples were also chromatographed with butanol-water. These chromatograms revealed only one slowly migrating component corresponding to deoxycytidine.

Several possibilities existed for the paradoxical behavior in butanol-boric acid chromatograms. One of these was that the variations in the patterns observed in these chromatograms were correct and that the butanol-water chromatograms failed to reveal these differences. Since the recovery of liver deoxyribosides (44 per cent) in the latter chromatograms was much lower than in butanol-boric acid chromatograms (78 to 86 per cent), it was at first suspected that thymidine was being destroyed in butanol-water. To test this possibility, 2 γ of thymidine were added to a liver concentrate and the mixture was chromatographed with butanol-water. In addition to the usual slow liver deoxycytidine-like band, a rapidly migrating band with the same R_F as thymidine and representing a recovery of 87 per cent of the added thymidine was observed. This experiment showed not only that thymidine was not destroyed by butanol-water but also that it could be separated from the other liver compounds in this solvent.

In a second experiment, a liver concentrate was chromatographed first with butanol-boric acid and then with butanol-water. In this chromatogram a rapidly migrating band was again observed as the main component, indicating that butanol-water did not destroy this component. In a final experiment, a liver concentrate, which contained a rapidly migrating component as the main deoxyriboside when chromatographed with butanol-boric acid, was chromatographed first with butanol-water and then with butanol-boric acid. This chromatogram showed only a single slowly moving band corresponding to deoxycytidine. Since butanol-boric acid failed to resolve butanol-water chromatograms further and since butanol-water failed to destroy or prevent the separation of added thymidine from liver extract, it would seem that the rapidly migrating band observed as a major component in the seven butanol-boric acid chromatograms was an artifact.

The low recoveries observed in butanol-water (Table IV) can be explained by the fact that the amount of growth in segments of the single band observed in these chromatograms exceeded the range in which growth was directly proportional to deoxynucleoside concentration. Such portions of the chromatogram would consequently give minimal values and low recoveries would result. Evidence that this was the correct explanation was provided by applying the usual amounts of liver concentrates to pieces of filter paper, moistening them with the solvent, and placing them in the chromatograph chamber while other strips were being chromatographed. After drying, the unchromatographed strips were soaked in water and the eluates were assayed microbiologically. Recoveries ranging between 90 and 100 per cent were obtained. In other experiments in which half as

much liver extract was chromatographed or the chromatograms were cut into 0.5 instead of 1.0 cm. strips, more adequate recoveries were observed in chromatograms with single bands.

Isolation and Identification of Blood and Liver Deoxyribosides—In order to identify the deoxyribosides further, much larger amounts of the liver and blood concentrates were chromatographed. In the first experiment, 3.0 ml. of blood concentrate and 6.0 ml. of liver concentrate were chromatographed twice with butanol-water. Portions of the chromatograms were examined microbiologically and with the cysteine-sulfuric acid reagent (12). Growth zones were observed at 0.34 to 0.45 R_F on the blood chromatograms and at 0.36 to 0.52 R_F on the liver chromatograms. The cysteine-sulfuric acid reagent reacted only with the blood chromatogram and produced a spot at 0.19 to 0.27 R_F which was devoid of microbiological activity and was not visible under ultraviolet light. The compound (or compounds) present in this portion of the blood chromatogram is still under investigation. The microbiologically active areas of the blood and liver chromatograms were cut out, eluted with water, and concentrated to 0.5 ml., and each was rechromatographed twice with butanol-water on a single paper strip. The results of rechromatography of these spots are shown in Table V (Experiment 1).

Visual examination of the chromatograms under ultraviolet light revealed two bands on the liver chromatogram and one on the blood chromatogram. The R_F of the blood spot (0.43) and of one liver spot (0.40) corresponded closely with those observed microbiologically (0.43 and 0.41, respectively). Furthermore, the zones not only reacted with cysteine-sulfuric acid, indicating the presence of deoxyribose, but their R_F values also agreed with those of pure deoxycytidine similarly determined. A rapidly migrating spot observed at 0.60 to 0.65 R_F on the liver chromatogram under ultraviolet illumination did not respond in the microbiological assay or the cysteine-sulfuric acid reaction and was not studied further.

The ultraviolet spectra of eluates of the microbiologically active areas of the blood and liver chromatograms were studied (Table V, Experiment 1). The spectral characteristics of the blood spot corresponded closely with those of pure deoxycytidine and the amount of deoxyriboside calculated to be present from the optical densities at 280 and 240 mμ (14.7 γ) also agreed with that found in the cysteine-sulfuric acid reaction (14.2 γ). Although an eluate of the liver spot exhibited an alkali shift in its spectrum characteristic of cytidine compounds, the absorption maxima and minima and the optical density ratios were different from those of pure deoxycytidine.

In Experiment 2, the liver deoxyribosides were resolved into two components as follows: A liver concentrate was chromatographed first with butanol-water and then with butanol-water-acetic acid. Microbiological

assay of a portion of the chromatogram revealed nucleoside bands at 0.50 to 0.59 R_F (Fraction A) and at 0.68 to 0.81 R_F (Fraction B). Fraction A accounted for about 60 per cent of the microbiological activity. The spots of Fractions A and B were cut out, eluted with water, and concentrated to a small volume, and each was rechromatographed on a single paper

TABLE V

Rechromatography of Rat Liver and Blood Extracts

The R_F values were obtained by dividing the distance traveled by the spot by the *mean* distance traveled by the solvent front. The range in R_F values is given in parentheses. 250/260 and 280/260 are ratios of the optical densities at these wavelengths.

Experiment No.	Material	Chromatographic spots			Spectral (mμ) characteristics of eluted spots					
		Ultraviolet illumination	Cysteine-sulfuric acid reaction	Microbiological assay	Maximum*	Minimum*	Maximum†	Minimum†	250/260	280/260
		R_F	R_F	R_F						
1	Liver	0.40 (0.35–0.45)	0.41 (0.37–0.45)	0.41 (0.34–0.48)	260	250	270	240	0.71†	1.00†
	Blood	0.43 (0.39–0.47)	0.43 (0.39–0.47)	0.43 (0.36–0.49)	270	255	280	240	0.39†	2.14†
	Deoxycytidine‡	0.42 (0.39–0.44)	0.41 (0.38–0.43)	Not determined	270	250	280	240	0.40†	2.04†
2	Liver Fraction A	0.49 (0.45–0.53)	0.49 (0.45–0.52)	0.48 (0.42–0.53)	260	240	265	235	0.93* 0.85†	0.51* 0.89†
	Liver Fraction B	0.52 (0.47–0.56)	0.51 (0.48–0.54)	0.52 (0.46–0.51)	270	255	280	250	1.19* 1.03†	0.88* 1.45†
	Deoxyuridine§	0.59 (0.56–0.61)	0.58 (0.55–0.61)	Not determined	260	240	265	235	0.93* 0.83†	0.45* 0.48†
	Deoxycytidine§	0.49 (0.47–0.51)	0.49 (0.46–0.51)	Not determined	270	255	280	250	0.96* 0.71†	0.92* 1.45†

* In 0.02 N NaOH.

† In 0.02 N HCl.

‡ Spectral characteristics determined on unchromatographed deoxycytidine.

§ Spectral characteristics determined on spots eluted from chromatograms.

strip with the same solvents as before. The results of rechromatography are given in Table V (Experiment 2). A single spot, whose position was identical when located microbiologically, under ultraviolet light, or with the cysteine-sulfuric acid reagent, was observed on the chromatogram of each fraction. Ultraviolet examination also revealed three other spots on the chromatogram of Fraction A and one other spot on the chromatogram of Fraction B which were inactive microbiologically and toward cysteine-sulfuric acid and were not studied further.

The ultraviolet spectra of eluates of the microbiologically active areas of the chromatograms showed that the positions of the absorption maxima and minima of Fractions A and B were identical with those of deoxyuridine and deoxycytidine, respectively. Three optical density ratios of Fraction A and two optical density ratios of Fraction B also corresponded to those of deoxyuridine and deoxycytidine, respectively. The amount of deoxyriboside found to be present in an aliquot of the Fraction A eluate by means of the cysteine-sulfuric acid reagent (8.0 γ) did not agree well with that calculated from the optical densities at the absorption maximum and minimum (20 γ). The deoxycytidine contents of Fraction B, determined in a similar manner, did not disagree as markedly (4.5 and 3.5 γ). The R_F of the Fraction A spot (0.49) was also considerably slower than that of pure deoxyuridine (0.59), although that of Fraction B (0.51) differed only slightly from that of deoxycytidine (0.49).

Intracellular Localization of Deoxyribosides—In view of the exclusive localization of DNA in the cell nucleus, it was of particular interest to determine the intracellular distribution of the deoxyribosides. This was done by centrifuging a 10 per cent homogenate of rat liver in isotonic saline for 10 minutes at 18,400 r.p.m. and 0°. It was found that the sediment, consisting of all the nuclei and mitochondria and some of the submicroscopic particles of the homogenate, contained 19 per cent of the total deoxyribosides while the supernatant liquid contained 81 per cent. On the basis of this experiment, it would appear that these compounds were present mainly in the non-particulate cytoplasm.

DISCUSSION

The data presented in this paper have clearly established the presence of deoxyribosides in tissue extracts. The most conclusive evidence for the identification of these compounds as deoxyribosides, apart from the specificity of the organism used for their detection and the similarity in chromatographic behavior of the tissue compounds and of the pure deoxyribosides, has been provided by the demonstration of a deoxyribose reaction on areas of paper chromatograms at which material absorbing ultraviolet light was observed and by the ultraviolet spectra of eluates of these zones. In the case of blood, the spectral and chromatographic data and the deoxyribose content of the eluate showed clearly that deoxycytidine was present (Table V).

From the spectral data on the isolated liver fractions (Table V), it is apparent that deoxyuridine and deoxycytidine must be major components of Fractions A and B, respectively, but the discrepancies between the optical density ratios of these fractions and of the pure compounds indicates the presence of other compounds as well. Nucleotides are excluded from

consideration as contaminants by their failure to migrate in the solvents employed and, since the absorption maxima and minima of Fractions A and B were the same as those of deoxyuridine and deoxycytidine, respectively, contaminants are further limited to those having the same or closely related absorption maxima and minima. On this basis, adenine, adenosine, and uridine would be possible contaminants of Fraction A, while cytidine could be present in Fraction B. The presence of one or more of the above compounds in Fraction A would readily account for the failure of the optical density ratios of this fraction to coincide with those of deoxyuridine, as well as for the fact that the amount of deoxyribose found in this fraction was less than that calculated from the difference in optical densities at the absorption maximum and minimum. In the case of Fraction B or the deoxycytidine fraction, the amount of deoxyribose found in the cysteine-sulfuric acid reaction was somewhat greater than that calculated from the difference in optical densities at 280 and 240 mμ. Further work will be required to determine whether the latter difference was significant, since the spectral measurements on Fraction B, although considerably more precise than those obtained with the deoxyribose reaction, agreed qualitatively but not quantitatively with those of pure deoxycytidine.

The discrepancies between the migration rate of Fraction A and deoxyuridine and of Fraction B and deoxycytidine (Table V) must also be noted. Although the R_F values of Fraction B and deoxycytidine differed only slightly, the R_F of Fraction A was much slower than that of pure deoxyuridine. Until sufficient amounts of these fractions become available to run mixed chromatograms with the pure compounds, the significance of the altered migration rates cannot be assessed.

The evidence presented here strongly suggests that deoxyribosides are normally present within the cell. The data on rat liver, for example, show that the total amount of deoxyribosides present is remarkably constant, regardless of whether the tissue was obtained under anesthesia or shortly after death. The deoxyribosides observed would consequently not seem to be products of DNA autolysis. If the latter were responsible for the presence of deoxyribosides in tissues, one would expect to find both purine and pyrimidine deoxynucleosides, but only the latter were present. Furthermore, deoxynucleotides might also be expected to be present in higher concentrations than deoxynucleosides, since they are earlier products of the catabolism of DNA than nucleosides. In contrast, however, our results have shown clearly that pyrimidine nucleosides are the main compounds present and that nucleotides and purine nucleosides, if they occur at all, must be present in much smaller amounts.

The absence of deoxynucleotides and of purine deoxyribosides suggests that the pyrimidine deoxyribosides in tissues function principally in the

synthesis of DNA. This view is well supported by the isotopic experiments of Reichard and Estborn (16) who found that, although deoxycytidine and thymidine were incorporated into DNA, deoxyhypoxanthosine was not. In addition, they observed that N^{15}-labeled deoxycytidine served as the precursor of either thymine or cytosine of DNA, whereas labeled thymidine served only as the precursor of thymine. It is, indeed, remarkable from the latter standpoint that deoxycytidine should prove to be a major component of the tissue deoxyribosides. Furthermore, the presence of deoxycytidine in blood suggests perhaps a circulating supply of this compound for the synthesis of DNA by certain tissues.

The presence of deoxyuridine in liver might at first be construed to indicate decomposition of DNA thymidine. Friedkin *et al.* (17)[4] have found, however, that deoxyuridine is a precursor of DNA thymidine in the chick embryo and in rabbit bone marrow. Since Reichard and Estborn (16) observed that deoxycytidine was a precursor of both thymine and cytosine in DNA, deoxyuridine would appear to be an intermediate formed from deoxycytidine and used in the synthesis of DNA thymidine. Such a mechanism for the synthesis of the pyrimidine components of DNA places deoxycytidine in a key position and suggests a search for enzymes involved in its formation and in its conversion to deoxyuridine and thymidine.

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SUMMARY

1. The presence of deoxyribosidic compounds in blood and in a wide variety of normal and tumor tissues of the rat and the mouse has been demonstrated with the aid of the organism, *Lactobacillus acidophilus* R-26, which specifically requires these compounds for growth.

2. The behavior of the tissue growth-promoting factors on ion exchange columns, on paper chromatograms, and during acid hydrolysis suggested that these compounds were pyrimidine deoxyribosides.

3. A microbiological method permitting quantitative determination of the separation of deoxyribosides on paper chromatograms was described and applied to rat liver and blood extracts and growth zones corresponding to deoxycytidine and deoxyuridine in migration rate were observed. In some liver chromatograms developed with butanol-boric acid, a band migrating at the same rate as thymidine was present in large amounts. Evidence was presented indicating that the appearance of this band was an artifact.

[4] Personal communication from Dr. M. Friedkin.

4. The deoxyribosides of rat liver and blood were isolated from large scale chromatograms and rechromatographed. It was shown that growth zones on the latter chromatograms coincided with spots observed under ultraviolet light or produced by the cysteine-sulfuric acid reaction for deoxyribose. The ultraviolet spectra of eluates of these spots indicated the presence of deoxycytidine in rat blood and of deoxycytidine and deoxyuridine in rat liver.

5. An unknown compound, devoid of the growth-promoting activity of deoxyribosides but reacting with cysteine-sulfuric acid, was observed on chromatograms of rat blood extracts.

6. Evidence for the localization of deoxyribosides in the soluble fraction of rat liver was presented.

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LACTOSE METABOLISM

IV. THE FUNCTION OF URIDINE TRIPHOSPHATE IN THE INTERCONVERSION OF GALACTOSE-1-PHOSPHATE AND GLUCOSE-1-PHOSPHATE*

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Enzymes provisionally termed "galactowaldenase" have been separated from yeast (1) and bacteria (2) which interconvert α-galactose-1-phosphate and α-glucose-1-phosphate with the catalytic participation of uridine diphosphohexose (UDPH). As yeast enzymes form a uridine galactose derivative from uridine diphosphoglucose, the following reaction sequence has been postulated (3) in which the over-all effect is an interconversion of the phosphate esters:

(1) UDPGal ⇄ UDPGlu

(2) Gal-1-P + UDPGlu ⇄ UDPGal + Glu-1-P

Sum, Gal-1-P ⇄ Glu-1-P

An alternative series of reactions has been postulated for this galactowaldenase (4). In addition to the above known constituents of the system, uridine triphosphate (UTP) and pyrophosphate (PP) have been implicated as follows:

(3) UTP + Gal-1-P ⇄ UDPGal + PP

(4) UDPGal ⇄ UDPGlu

(5) UDPGlu + PP ⇄ UTP + Glu-1-P

Sum, Gal-1-P ⇄ Glu-1-P

This reaction sequence requires a direct participation of UTP and PP with each molecule of ester interconverted. Evidence for Reactions 3 and 5 has recently been presented (5).

Additional information relative to these reactions has been obtained with fractionated extracts of *Saccharomyces fragilis* and *Lactobacillus bulgaricus*. Unfractionated, cell-free extracts readily interconvert glucose-1-phosphate and galactose-1-phosphate in the presence of catalytic amounts of either

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UDPH or UTP. Fractionation of enzymes from crude extracts has further clarified the function of UTP and has eliminated the alternative reaction sequence as functioning in the galactowaldenase reactions.

Methods

The preparation of crude extracts of *L. bulgaricus* was described in a previous publication (2). The fractionation procedure for *L. bulgaricus*, begun with heat treatment of the extract at pH 5, ammonium sulfate precipitation, and dialysis (6), has given the galactowaldenase system (Reactions 1 and 2) free of "uridyl transferases" (Reactions 3 and 5).

The yeast extracts were made from dried *S. fragilis* obtained from the National Dairy Research Laboratories.[1] 1 volume of dried yeast was extracted with 2 volumes of ammonium phosphate buffer at pH 8.2 for 24 hours, the suspension was centrifuged, and the supernatant fluid dialyzed against several changes of distilled water at 4°. The galactowaldenase system of this yeast preparation was stable when held at freezing temperatures, but repeated freezing and thawing reduced the concentration in the extract. The uridyl transferase activity in yeast was stable under these conditions.

Enzymatic activity was followed by measuring stabilization of acid-labile phosphate as described previously (2). UDPH, prepared from an extract of yeast and containing both the glucose and galactose derivatives, was essentially free of UTP; however, it contained other nucleotides, principally adenylic acid. The UDPH was therefore further purified on a Dowex (formate) column (7). The UTP was a commercial preparation obtained from the Pabst Laboratories.

Results

Unfractionated Extracts—When the crude extracts of *L. bulgaricus* were incubated in the presence of UDPH, the α-galactose-1-phosphate isomerized was a linear function of time (Fig. 1). However, when UTP was added to a similar system without UDPH, there was an initial lag period, followed by activity which closely paralleled that obtained with UDPH. These data suggest that UDPH, the coenzyme for this isomerization, was limiting in the early phases of the incubation with UTP, and, in addition, the active UDPH coenzyme may be formed by enzymes which are present in the crude preparation from UTP and hexose ester.

Fractionated Extracts—The fractionation of *L. bulgaricus* as outlined produced an enzyme which was activated by UDPH as expected, but which UTP failed to activate (Tubes 1 and 2, Fig. 2). On the other hand, when yeast extract was frozen and thawed successively, it was possible to obtain

[1] Oakdale, Long Island.

an enzyme which would not isomerize α-galactose-1-phosphate in the presence of either UDPH or UTP (Tubes 3 and 4). Fractionated *L. bulgaricus* enzyme and *S. fragilis* enzyme in combinations readily isomerized α-ga-

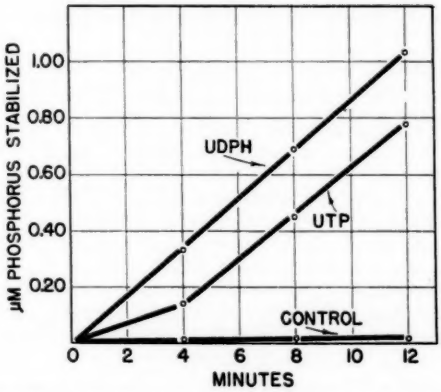

FIG. 1. Isomerization of galactose-1-phosphate with unfractionated extracts of *L. bulgaricus*. The control tube contained 0.2 ml. of buffer (pH 7.1), Mn^{++} 0.01 M, α-galactose-1-phosphate 0.02 M, phosphoglucomutase extract, water to make a total final volume of 1.4 ml., and 0.2 ml. of *L. bulgaricus* extract. UTP and UDPH were added at the expense of water as indicated.

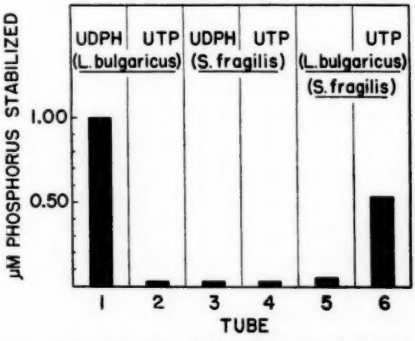

FIG. 2. Isomerization of α-galactose-1-phosphate by fractionated extracts. The reaction time was 10 minutes, and the experimental conditions and additions to the various tubes were similar to those in Fig. 1, with the exceptions noted.

lactose-1-phosphate when UTP was present, thus providing additional evidence that UTP is functioning in the formation of the UDPH. To lend additional confirmation to this observation, the yeast enzyme and UTP were preincubated with either glucose-1-phosphate or galactose-1-phos-

phate. The products formed from both of these incubations were then found to stimulate the fractionated *L. bulgaricus* enzyme (Table I).

TABLE I

Isomerization of Galactose-1-phosphate with Fractionated L. bulgaricus Extracts

Each tube contained 0.1 ml. of buffer (pH 7.1), Mn^{++} 0.01 M, α-galactose-1-phosphate 0.02 M, phosphoglucomutase. Water or the activator was added to a total final volume of 0.7 ml., and then *L. bulgaricus* extract.

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
	Water	UDPH	UTP	Sample A*	Sample B*
Phosphorus stabilized, *μmole*	0	0.73	0	0.53	0.50

* Prepared by incubating 0.2 μmole of α-galactose-1-phosphate (Sample A) and glucose-1-phosphate (Sample B) each with 0.2 μmole of UTP and *S. fragilis*. The reaction was stopped by boiling for 30 seconds. After centrifugation, an aliquot of each was added to Tubes 4 and 5, respectively.

DISCUSSION

This study has provided confirmation for the experiments of Munch-Petersen *et al.* (5) in that UDPH is formed from UTP and hexose-1-phosphate by enzymes present in yeast (Reactions 3 and 5 above). In addition, bacteria (*L. bulgaricus*) also can achieve formation of UDPH in a similar manner.

When UDPH is formed from UTP and hexose phosphate, the interconversion of the hexose esters is possible without further participation of UTP. Thus the partial separation of enzymes provides conclusive evidence eliminating UTP and PP participating in the galactowaldenase reaction, as postulated by Novelli and Soodak (4). Even though Reactions 3 and 5 are consistent with available data, they should not be construed to play a direct rôle in the galactowaldenase reaction. This can still be visualized as at least a two-step process not involving UTP and PP, as in Reactions 1 and 2 above. Leloir (3) has provided evidence for Reaction 1. While the postulated transfer Reaction 2 is consistent with data of Kalckar *et al.* (8) and Trucco (9), it is in need of more complete resolution. Further purification of the *L. bulgaricus* enzymes should be helpful.

SUMMARY

1. Unfractionated extracts from *Lactobacillus bulgaricus* and *Saccharomyces fragilis* interconvert galactose-1-phosphate and glucose-1-phosphate when catalytic amounts of either uridine diphosphate hexose or uridine triphosphate are present.

2. Fractionated extracts of *L. bulgaricus* respond only to uridine diphosphate hexose.

3. Uridine diphosphate hexose was formed from either galactose-1-phosphate or glucose-1-phosphate, and uridine triphosphate from enzymes present in the *S. fragilis* extracts and in the unfractionated *L. bulgaricus* extracts.

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COLORIMETRIC DETERMINATION OF CARBON MONOXIDE IN AIR BY AN IMPROVED PALLADIUM CHLORIDE METHOD*

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In the absence of other reducing substances the following reaction is an old test for the presence of carbon monoxide (1):

$$PdCl_2 + CO + H_2O = Pd + CO_2 + 2HCl$$

The decrease in palladium chloride can be determined colorimetrically (2) and by means of iodometric titration (3). Christman, Block, and Schultz (4) determined CO in air by transferring gas mixtures to flasks at pressures of about 0.5 atmosphere, containing known quantities of $PdCl_2$. After 6 to 24 hours the $PdCl_2$ which remained was converted to PdI_2 by the addition of excess KI, the pinkish red color being compared with that of standard solutions. Instead of a colorimeter we have used a Beckman, model DU, spectrophotometer. Many accurate determinations with small quantities of reagents are easily performed in less than 3 hours. By means of special stop-cocks which fit into standard volumetric glassware of measured capacity it is possible to evacuate with a water pump and then to introduce a gas. The results on concentrated and dilute CO mixtures show that the improved method has great flexibility.

According to previous reports it is not possible to recover CO completely by the $PdCl_2$ method (2–4). This is probably the result of the following reaction,

$$Pd + \tfrac{1}{2}O_2 + 2HCl = PdCl_2 + H_2O$$

for a 20 to 25 per cent lower recovery of CO occurs in O_2 than in N_2 mixtures (4). In recognition of the above opposing reactions, we have investigated the consistency of recovery at different times after the reaction was started and have found at the end of the 2nd hour that recovery is 99.6 per cent in the presence of air at atmospheric pressure and at room temperature.

An excellent comparison of six other methods was reported by Shepherd (5), who prepared dilute CO mixtures and then distributed these in steel cylinders to various laboratories for analysis. We were fortunate in obtaining a similarly prepared mixture which was analyzed by the improved

* This study was supported in part by a grant from Eli Lilly and Company.

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$PdCl_2$ method with results that compare favorably with those by the best methods available in 1947.

Materials

Palladium Chloride[1]—With a semimicro balance 125 to 130 mg. of 99.7 per cent pure $PdCl_2$ are weighed to the nearest 0.1 mg. in a 20 ml. beaker. This is then placed in a 500 ml. beaker and 100 ml. of distilled H_2O and 1 ml. of HCl solution are added. The latter is prepared by diluting concentrated HCl solution ten times with distilled H_2O. The beakers are covered with a bell jar and left at room temperature until the $PdCl_2$ dissolves, which takes about 12 hours. The solution is then transferred quantitatively to a 250 ml. volumetric flask and is brought to volume with distilled

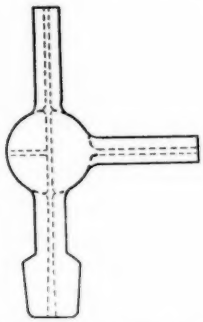

FIG. 1. Removable stop-cock for insertion into reaction vessels

H_2O. The stock solution is stored at room temperature in 125 ml. reagent bottles with glass stoppers and can be used for at least 8 months.

Aluminum Sulfate—A 10 per cent solution of $Al_2(SO_4)_3$ in distilled H_2O is kept in a dropping bottle.

Potassium Iodide—A solution of reagent grade, granular KI is prepared daily in the proportions of 12 gm. of KI and 100 ml. of distilled H_2O, with a pan balance and a graduated cylinder.

Glassware—A few pieces of special glassware are necessary. Fig. 1 shows a modified Geissler type stop-cock.[2] The lower arm has an interchangeable ground joint which is greased and inserted into the standard taper, No. 13, neck of volumetric vessels, such as 25 ml. graduated cylinders and 100 and 200 ml. volumetric flasks. The capacity of each reaction vessel is com-

[1] Palladium chloride of assayed purity can be purchased from A. D. Mackay, Inc., 198 Broadway, New York 38.

[2] Made according to specifications by Metro Industries, 29-28 Forty-first Avenue, Long Island City 1, New York.

puted from the weight and the temperature of the water which fills the vessel and the capillary in the stop-cock. Among the wide choice in size of calibrated vessels, cylinders of 40 ml. capacity are used most often.

At the end of a test the solutions are removed in dropping pipettes with rubber nipples and are transferred to standard Corning, coarse fritted, Büchner type filters of diameter 10 mm. The capacity of the filters is enlarged from 2 to 8 ml. by increasing the height of the funnel. The filtrate is usually collected in 50 ml. volumetric flasks. In tests with very dilute CO, the filtrate from a 2 ml. funnel is collected in 10 ml. volumetric flasks.

All glassware is cleaned by removing the metallic Pd with a few drops of chromic-sulfuric acid mixture, followed by rinsing with water.

CO Mixtures—Highly concentrated CO was obtained in steel cylinders[3] at a pressure of 800 pounds per sq. in.

A series of dilutions was prepared by mixing CO and room air. The CO was made from formic acid and sulfuric acid, and was analyzed for purity (6). A tonometer was connected to a Douglas bag, and the contained CO flowed into the bag along with a measured volume of room air that issued from a calibrated Tissot spirometer. The gases were thoroughly mixed by beating the bag, and the analysis was performed immediately.

A 0.0382 per cent CO mixture was kindly supplied by the National Bureau of Standards through the courtesy of Dr. Shuford Shuhmann, who informs us that this mixture could be 0.0378 per cent or 0.0386 per cent as judged by independent calibration of pressure gages (5).

Procedure

Fig. 2 shows a cylinder containing a known volume, usually 1.00 ml. of $PdCl_2$ solution, and 1 drop of 10 per cent $Al_2(SO_4)_3$ connected to an Hg manometer and to a water pump. The apparatus is evacuated to a desired extent, and the Hg level is noted. Then the stop-cock (Fig. 1) is turned, closing the cylinder and connecting the manometer to room air. The Tygon[4] tubing is removed from the vertical arm of the stop-cock, which is then connected to a rubber tube from a Douglas bag, or a rubber bladder, containing the CO mixture to be analyzed. The horizontal arm of the stop-cock is connected to a rubber tube that leads into an inverted 1 liter graduated cylinder which is filled with H_2O. By pressing on the Douglas bag and then opening its screw clamp, a sufficient volume of gas is expressed so that the connections are flushed with at least 10 times their volume. Manual pressure on the bag is removed, and then the stop-cock is turned so that the gas mixture enters the cylinder. After 15 seconds the cylinder

[3] Matheson Company, Inc., East Rutherford, New Jersey.
[4] U. S. Stoneware, Akron 9, Ohio.

is closed, and the barometric pressure and room temperature are noted. All told, this takes about 2 minutes.

It is necessary to know roughly the CO content and to have in the reaction vessel at least 10 per cent more $PdCl_2$ than is required; this can be determined from the fact that 0.1261 ml. of CO reduces 1 mg. of $PdCl_2$. With entirely unknown mixtures, it is wise to make a series of tests on successively larger gas samples. With 0.010 per cent CO or less, the stock

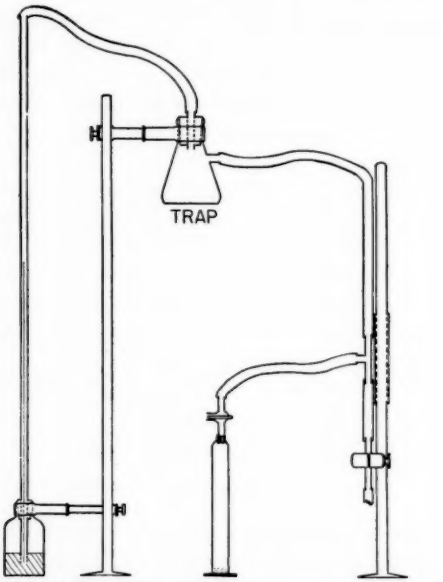

FIG. 2. Apparatus for introduction of gas samples into reaction vessels

solution is diluted five times, or the cylinders can be replaced with larger volumetric flasks.

When the gas has been introduced, the cylinder is gently rotated by hand and is then placed horizontally without, at the same time, permitting the fluid to enter the capillary of the stop-cock. At the end of 2 hours at 20–24° the mixture of $PdCl_2$ and precipitated Pd is transferred to a filter. The filtrate is collected in a 50 ml. volumetric flask which contains 20 to 30 ml. of distilled H_2O. The cylinder is washed with two portions of 2 to 3 ml. of distilled H_2O, which are transferred to the filter. Then from a 10 ml. pipette, containing 12 per cent KI solution, about 3 ml. are placed

on the filter, and the rest is emptied directly into the volumetric flask. After this the filter is washed with 2 to 3 ml. of distilled H_2O, the flask is brought to volume with distilled H_2O, and the contents are thoroughly mixed. When stock $PdCl_2$ solution diluted five times is used, the same procedure is followed, except that 2 ml. filters drain into 10 ml. volumetric flasks and 2 ml. of 12 per cent KI are placed directly on the filters.

Standard solutions are prepared in triplicate with the same quantities of reagents that are used in the tests. These are placed in 50 or in 10 ml. volumetric flasks. At the time that the tests are filtered, the KI solution is added to the standards and these are brought to volume with distilled water.

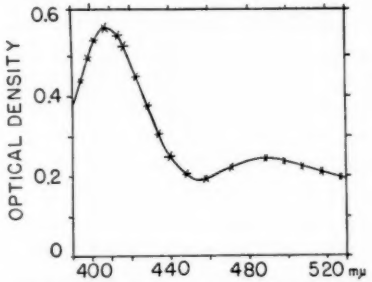

FIG. 3. Spectral absorption of PdI_2 prepared by mixing 0.0998 mg. of $PdCl_2$ in dilute HCl and 1 drop of 10 per cent $Al_2(SO_4)_3$ with approximately 240 mg. of dissolved KI and diluting with H_2O to 10 ml.

The standard and test solutions are placed in Pyrex cuvettes of 1 cm. light path and read against a water blank in a Beckman, model DU, spectrophotometer at 490 mμ. The spectral absorption of PdI_2 in these solutions is shown in Fig. 3 (see the legend). Although the light absorption at 408 mμ is greater than at 490 mμ, the former gradually increases by 20 per cent in the course of 24 hours, whereas the latter develops with rapidity, is extremely stable, and is unaffected by greatly increasing the concentration of KI.

The volume of CO (normal temperature and pressure, dry) in a test is computed as follows:

$$\left[Q - \left(\frac{D_t}{D_s} \times Q\right)\right] 0.1261$$

where Q is the mg. of $PdCl_2$ originally present, D_t is the optical density of the test solution, and D_s is the mean optical density of three standard solutions. 0.1261 ml. of CO (normal temperature and pressure, dry) is equivalent to 1.00 mg. of $PdCl_2$.

The volume of the gas sample (normal temperature and pressure, dry) drawn into the reaction vessel is computed as follows:

$$(C - V) \times \frac{\Delta P}{P} \times \frac{P - p}{760} \times \frac{273}{273 + T}$$

C is the ml. capacity, and V is the ml. volume of reagent solutions in the reaction vessel; ΔP is the rise in mm. of Hg in the manometer connected to the vessel, and P is the atmospheric pressure (it is necessary to correct the manometric and barometric readings for temperature effect on the scales); p is the vapor pressure of water at room temperature; T is the temperature of the room and reaction vessel.

Concentrated CO—Industrial CO was collected over CO-saturated water. A representative gas sample was drawn into a burette of 0.128 ml. capacity, and the burette was connected through a rubber stopper to a 50 ml. Erlenmeyer flask containing 5.00 ml. of stock $PdCl_2$ solution. The stop-cock on the burette was opened, and the gas was introduced by means of an Hg leveling bulb. 2 hours later the test solution was filtered into a 100 ml. volumetric flask to which 20 ml. of 12 per cent KI were added.

CO in H_2O—As a control on the transfer of CO in gas mixtures, distilled H_2O was equilibrated with 96 per cent CO in tonometers from which 5.00 ml. portions were taken for gasometric determinations (7) and 2.00 ml. portions for measurement with $PdCl_2$. The aqueous solution was delivered directly into 5.00 ml. of dilute $PdCl_2$ stock solution in a 40 ml. cylinder, which was immediately closed with a greased stopper.

RESULTS AND DISCUSSION

Early in these experiments we found that $PdCl_2$ is rapidly reduced by CO, which is already in solution. Comparison of the results obtained by the gasometric and $PdCl_2$ methods shows (Table I) that the oxidation of dissolved CO was practically complete in 20 minutes. For the next 3 hours there was little difference in the results.

When CO must diffuse from a gas to a liquid phase, a longer period of time is required for the reduction of $PdCl_2$. To oxidize 90.8 per cent of the CO, as shown in Table II, 0.5 to 1.0 hour is required. The reaction is probably completed in 2 hours, but the corresponding reduction of $PdCl_2$ is complicated by a slow oxidation of Pd, as judged by progressively decreasing recoveries which fall from 98.1 per cent in 2 to 5 hours to 91.1 per cent in about 24 hours and 87.1 per cent in 48 hours. A similar effect on recovery of CO was observed by Wennesland (3).

The standard solutions served as controls for all steps except for the introduction of gas samples and the filtration of solutions. These controls (Table II) have a standard deviation of 0.90 per cent compared with 2.28

per cent for recovery, and hence the recovery of 98.1 per cent is not significantly different from 100.0 per cent. Moreover, the recovery was computed for 0.0382 per cent CO; so, if the gas were 0.0378 per cent, the recovery would be 99.1 per cent. Although the purity of the $PdCl_2$ was 99.7 per cent, this being less than 100.0 per cent does not improve the recovery,

TABLE I

Recovery of CO Present in 2.00 Ml. of H_2O at Intervals after Mixing with $PdCl_2$

Gasometric determinations carried out as described by Roughton and Root (7). Recovery is computed by taking the gasometric determinations as 100 per cent.

Reaction period	$PdCl_2$	CO, gasometric	Recovery
hrs.	*vols.*	*per cent*	*per cent*
0.3	1.79	1.82	98.2
1.0	1.98	1.95	101.5
1.5	1.84	1.82	101.1
1.5	1.95	1.91	102.1
1.7	1.98	1.95	101.5
2.3	1.83	1.82	100.5
3.0	1.92	1.95	98.4
Mean			100.5

TABLE II

Recovery of 0.0382 Per Cent CO at Different Periods after Reaction with $PdCl_2$

Reaction period	No. of determinations	Degrees of freedom	Recovery	Standard deviation
hrs.			*per cent*	*per cent*
0.5- 1.0	11	10	90.8	4.69
2 - 5	16	15	98.1	2.28
17 -25	23	22	91.1	2.60
48	2	1	87.1	1.49
Control	36	24	100.0	0.90

because the standard and test solutions were not corrected for the slight impurity.

At intervals along the ordinate in Fig. 4 are listed the various expected concentrations of CO, and opposite each interval the percentage recovery is plotted. In the right-hand border of Fig. 4 are typical sample volumes and initial quantities of $PdCl_2$. All tests with the $PdCl_2$ method ran for about 2 hours.

The first mixture (Fig. 4) is 95.1 per cent, as this is the mean of eight determinations with a microgasometric procedure (6). All of the four

determinations with $PdCl_2$ are in close agreement and attest to the reproducibility of the method.

The next five mixtures (Fig. 4), from 0.258 to 0.0392 per cent, were analyzed with $PdCl_2$ and a gasometric method (7) in which reduced hemoglobin is employed. The gasometric results generally agree with those expected from the dilution of CO with room air. The gasometric results on 0.091 per cent CO are low, and, were it not for the $PdCl_2$ results, would lead one to suspect that a mistake had been made in diluting the gas.

The 0.0152 and 0.0103 per cent mixtures (Fig. 4) were analyzed by a gasometric method with 300 ml. samples (7). During the analysis of the

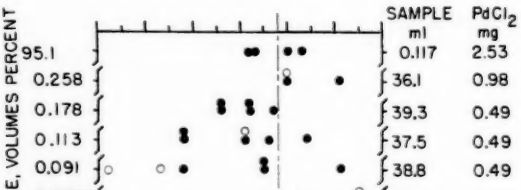

FIG. 4. Results obtained in 2 hour tests with the improved $PdCl_2$ method upon industrial CO, a series of mixtures prepared in Douglas bags, and a mixture prepared by the National Bureau of Standards. ○ = gasometric method, ● = $PdCl_2$ method.

most dilute mixture an excessive quantity of O_2 may have been introduced, thus causing the gasometric results to be low and unreliable. Though slightly inconsistent, the results on the 0.0103 per cent CO with $PdCl_2$ are acceptable. Since the time when these measurements were performed with the two dilute mixtures, we have obtained better results by testing with one-fifth the indicated quantity (Fig. 4) of $PdCl_2$. For example, in experiments with 20 ml. of blood (8) in 1 liter bottles we have obtained good recovery with 10 ml. gas samples from CO mixtures as low as 0.0010 volume per cent. From these various points of view we believe that the gas samples were obtained without the introduction of a constant error and that in a 2 hour reaction period recovery is nearly perfect.

When air which is to be examined for CO also contains substances such as acetylene, ethylene, and H_2S, these can be removed by scrubbing with

Br_2 water and KOH solutions (4). We have tested the saturated volatile substances, acetone, alcohol, and ether, which could be encountered in physiological experiments and, of course, have found that these do not reduce $PdCl_2$. Since Hg displaces Pd in the electromotive series, we warn that care must be taken to see that globules of Hg do not contaminate the $PdCl_2$ solutions.

SUMMARY

Christman's method of analyzing CO in air by the reduction of $PdCl_2$ has been extensively improved so that it is suitable for use in physiological experiments. With samples of less than 100 ml. it is possible to analyze CO percentages of 94.2, 0.010, and even less. The accuracy and consistency of the method are shown by recovery of 99.6 per cent, with a standard deviation of 2.21 per cent. The lowermost limit of the method, which has not been approached, has to do with the least quantity of $PdCl_2$ that can be accurately measured by spectrophotometry of PdI_2 solutions.

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AN IMPROVED PALLADIUM CHLORIDE METHOD FOR THE DETERMINATION OF CARBON MONOXIDE IN BLOOD*

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The improved palladium chloride method used for the measurement of carbon monoxide in air mixtures (1) has been adapted for the determination of CO in blood. The principle of the method consists in releasing CO from blood by the addition of a ferricyanide-saponin solution. CO diffuses at atmospheric pressure into a second reaction flask where at room temperature $PdCl_2$ is reduced to metallic palladium (2). The addition of an excess of KI converts unreduced $PdCl_2$ to soluble PdI_2, and the pinkish color of the latter is compared with that of a standard solution.

With this procedure measurements of the CO content of 0.20 ml. samples of blood agree well with precise gasometric determinations (3) carried out upon 1.0 ml. of the same blood. The new palladium method, which requires equipment available in many laboratories, is less laborious and requires less skill than the gasometric technique. Moreover, smaller samples of blood are needed, and in the same length of time more measurements can be carried out.

EXPERIMENTAL

Reagents—In addition to the reagents used for the determination of CO in air mixtures (1), a ferricyanide-saponin solution is required. This consists of a mixture of 2 gm. of saponin and 1 gm. of $K_3Fe(CN)_6$ and 100 ml. of distilled H_2O. The solution is prepared daily.

Procedure—Only 0.2 ml. of blood is required for the analysis of the CO content of blood containing more than 1 volume per cent of CO. Two 25 ml. Erlenmeyer flasks[1] with standard taper ground glass joints are used (Fig. 1). In one of these 1.0 ml. of ferricyanide-saponin solution is placed. The second reaction flask contains 1 drop of $Al_2(SO_4)_3$ solution and 1.00 ml. of $PdCl_2$ solution which is always freshly prepared every 3rd day by diluting 5.00 ml. of stock $PdCl_2$ (1) to 25 ml. with distilled H_2O. A calibrated Folin blood pipette is used to measure accurately 0.200 ml. of heparinized

* This study was supported in part by a grant from Eli Lilly and Company.

[1] Made according to specifications from medium length joints 14/20, by Metro Industries, 29-28 Forty-first Avenue, Long Island City 1, New York.

blood, which is delivered under the surface of the ferricyanide-saponin solution, the pipette being rinsed with the solution. Immediately, the two flasks are connected without mixing their contents, and the greased joints are sealed by rotation. The flasks are linked with a lightly stretched rubber band and inserted into metal clips on a wheel which is rotated by an electric clock motor at 8 r.p.m. for 3 hours.

The contents of the $PdCl_2$ flask are transferred with a dropping pipette to a funnel and filtered through a coarse fritted disk into a 10 ml. volumetric flask. The reaction flask is rinsed with two successive 2 ml. portions of distilled H_2O, which are transferred to the filter. Then, 2.0 ml. of 12 per cent KI solution are placed in the filter, the latter is washed with distilled H_2O, and the flask is brought to volume and mixed thoroughly.

The optical density at 490 mμ of the solution is compared with that of standard solutions prepared as described in the method for determination of CO in air mixtures (1).

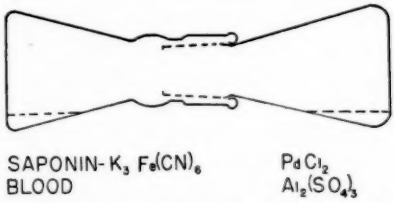

FIG. 1. Blood carbon monoxide reaction vessels (2)

Calculations—The volume per cent of CO in blood is computed from the equation

$$\left[Q - \left(\frac{D_t}{D_s} \times Q\right)\right]\left[\frac{0.1261 \times 100}{V}\right]$$

in which Q is the quantity in mg. of $PdCl_2$ originally present, D_t is the optical density of the test solution of PdI_2, and D_s is that of the standard solutions. According to the chemical equation of the reaction of CO with $PdCl_2$ (1), 22.4 ml. of dry CO at 0° and 760 mm. of Hg will reduce 177.6 mg. of $PdCl_2$. Therefore, 1 mg. of $PdCl_2$ is equivalent to 0.1261 ml. of CO, and this is multiplied by 100 to give volume per cent of CO, where V is the volume of blood sample in ml.

Changes in Procedure with Low and High CO Contents of Blood—Animals and non-smokers have low, but detectable, quantities of CO (0.05 to 0.35 volume per cent) in their blood (4). For this analysis 1.00 ml. of heparinized blood and 2.5 ml. of ferricyanide solution in 50 ml. reaction flasks are used (Table I).

When the CO capacity of blood is determined, the amount of $PdCl_2$ must be increased 5-fold, and therefore 1.00 ml. of stock $PdCl_2$ is employed. About 20 ml. of distilled H_2O are used to transfer the $PdCl_2$ solution and rinse the reaction flask (1), the filtrate being collected in a 50 ml. volumetric flask (Table I). To the volumetric flask are delivered 8 ml. and to the filter 2 ml. of 12 per cent KI, after which 4 to 6 ml. of distilled H_2O are run through the filter, and the filtrate is brought to volume. Standard so-

TABLE I

Determination of CO in Blood

Sample A, $PdCl_2$ method; Sample B, gasometric method. The quantities of $PdCl_2$ and KI, the size of the reaction flasks, and the final volumes of filtrates used for the determination of different concentrations of CO are indicated.

Blood sample		$PdCl_2$	Volume of each reaction flask	Saponin-ferri-cyanide	Filtrate volume	Quantity of KI	Blood CO
	ml.	*γ*	*ml.*	*ml.*	*ml.*	*gm.*	*per cent*
A	1.0	99.8	50	2.5	10	0.24	0.13, 0.15, 0.16, 0.16
B	2.0						0.11
A	0.2	99.8	25	1.0	10	0.24	1.02, 1.05, 1.09
B	1.0						1.05, 1.07
A	0.5	99.8	25	1.0	10	0.24	1.30, 1.36, 1.38
B	1.0						1.35, 1.39
A	0.2	99.8	25	1.0	10	0.24	2.44, 2.47, 2.48, 2.55, 2.56
B	1.0						2.45, 2.50, 2.53, 2.58
A	0.2	99.8	25	1.0	10	0.24	3.99, 4.01, 4.01, 4.04, 4.04, 4.08
B	1.0						4.02, 4.06
A	0.2	253	25	1.0	10	0.24	10.2, 10.4, 10.4, 10.5
B	1.0						10.3, 10.5
A	0.2	499	25	1.0	50	1.2	18.4, 18.4, 18.4, 18.4, 18.7
B	1.0						17.7, 18.0, 18.3, 18.7

lutions are prepared in triplicate with 1.00 ml. of stock $PdCl_2$, 1 drop of $Al_2(SO_4)_3$, 10 ml. of 12 per cent KI, and H_2O to a total of 50 ml.

RESULTS AND DISCUSSION

More than 600 measurements of the CO content of blood have been carried out on the blood of rats, dogs, and men; the accuracy of the results is always compared with results by the Roughton and Root method (3). At first in this investigation we used, as recommended by Wennesland (2), a few drops of 10 per cent H_2SO_4 to release the CO bound to hemoglobin. We always found the results obtained with the $PdCl_2$ method to be 0.1 to 0.2 volume per cent higher than those with the gasometric technique.

However, when we used the ferricyanide-saponin mixture described above, there was good agreement between the results obtained with the two methods.

As will be noted in Table I, in which are set forth the results of a few typical experiments, the $PdCl_2$ method requires a blood sample of 1.0 ml. at the low levels of CO present in the blood of animals and non-smoking men. CO measurement of such blood by the gasometric method can be carried out upon 1.0 or 2.0 ml. of blood, but slightly better results are obtained with the larger quantity.

When the CO is 1.0 to 4.0 volume per cent, excellent agreement is found between the results obtained by the $PdCl_2$ and gasometric methods (Table I). These are the levels of CO usually found in physiological experiments having to do with the volume distribution of CO in blood (4, 5). It should be noted that in these experiments the $PdCl_2$ determinations were carried out with 0.2 ml. samples, whereas the gasometric method required 1.0 ml. of blood.

In Table I are also shown determinations on blood which has a dangerously high, if not fatal, level of CO, and again the results obtained with the $PdCl_2$ and gasometric methods agree closely. Good agreement was also found for measurement of CO capacity.

A 3 hour period of rotation was used because, as shown in the preceding paper (1) dealing with the measurement of CO in air mixtures, nearly complete recovery of the CO present was obtained in 2 hours. Visual observation of the color of blood when placed in ferricyanide-saponin solution indicates that about 5 minutes are required for development of the brown color of methemoglobin. Another period of 10 minutes elapses before the first traces of precipitated palladium appear. To be on the safe side we have arbitrarily chosen 3 hours as the period of rotation. Experiments of shorter duration, as of 1 hour, give results which are too low by some 10 per cent.

To test the completeness of extraction of CO from the blood-ferricyanide-saponin mixture, we have removed the flask containing $PdCl_2$ solution at the end of 3 hours and substituted a second flask containing $PdCl_2$ solution. In all instances there was no evidence that any additional CO diffused from the blood mixture. We assume, therefore, that the extraction of CO is complete in 3 hours.

The determinations of CO made by the $PdCl_2$ method and reported in Table I were obtained under our direction by a competent technician who had completed the usual undergraduate courses in chemistry. One of us measured the blood and delivered it from the pipette. All other steps were carried out by the technician. On the whole the consistency of the results is excellent and is about as good as is permitted by the combined

error of the procedures. The method is not laborious, for although 3½ hours are required for the entire procedure, only ¾ of an hour is spent in technical procedures. It should be possible for one person to make at least eighteen determinations in one working day. Other than the use of great care in preparing the stock solution, the details which require attention are in the use of pipettes and in the quantitative transfer and filtration of solutions. The best index of the accuracy of pipetting and photometry is the consistency of the optical density readings of the three standard solutions (1).

We are indebted to Miss Leah Geller for technical assistance.

SUMMARY

An improved $PdCl_2$ method for the determination of CO in 0.2 ml. to 1.0 ml. blood is described. The results agree well with those obtained with a precise gasometric method.

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ON THE ORIGIN OF ANIMAL ERGOTHIONEINE*

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Studies in this Laboratory with isotope-labeled compounds have given no evidence for the synthesis of ergothioneine by the rat, chicken, guinea pig, or human (1). On the other hand Heath and coworkers (2) have concluded from similar experiments that the boar does synthesize ergothioneine. We now report the results of studies on a young pig, in which we find no evidence for ergothioneine synthesis.

Closely related to the question of ergothioneine synthesis by animals is the effect of dietary constituents on the levels of blood ergothioneine. Eagles and Vars (3) several years ago showed that diets containing corn led to increased blood ergothioneine in pigs, and Potter and Franke (4) observed similar effects with rats. In contrast, Hunter (5) was unable to find any marked effect of corn diets on blood ergothioneine in either pigs or rats. Moreover, several attempts to identify ergothioneine as a constituent of corn or other plant material have failed (5–7). In this paper we present evidence to show that corn can serve as a dietary precursor of blood ergothioneine in rats and also that the dietary level of ergothioneine required to produce this effect is so small that it may well explain failures to detect ergothioneine in plant material.

EXPERIMENTAL

Feeding of S^{35}-Methionine to Pig—The experiment was patterned as closely as possible after that described by Heath and coworkers (2). The available facilities necessitated the use of a small animal; therefore an 8 week-old boar (Chester white breed) was used. This animal had been weaned at 6 weeks and was maintained thereafter on a diet consisting of cracked corn 200, bran 100, middlings 100, meat scraps 50, bone meal 50, oil meal 50, NaCl 5, charcoal 5, and powdered skimmed milk 25 parts. A blood ergothioneine determination (8) showed a level of 14.5 mg. per 100 ml. A solution of 192 mg. of L-methionine containing 1 mc. of S^{35} (Schwarz Laboratories, Inc., Mt. Vernon, New York) was mixed with small portions of the food and administered over a period of 4 days. 3 weeks later the

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animal was anesthetized and as much blood as possible (1200 ml.) was collected from the severed neck vessels. The procedure devised by Williamson and Meldrum (9) for the isolation of ergothioneine from pig blood was applied to the blood sample. Unexpected losses of ergothioneine were encountered, and the final product after precipitation and decomposition of the copper salt was highly impure. The material was purified further by precipitation as the phosphotungstate (2); decomposition of this salt yielded 35 mg. of solids containing 12.8 mg. of ergothioneine and having a

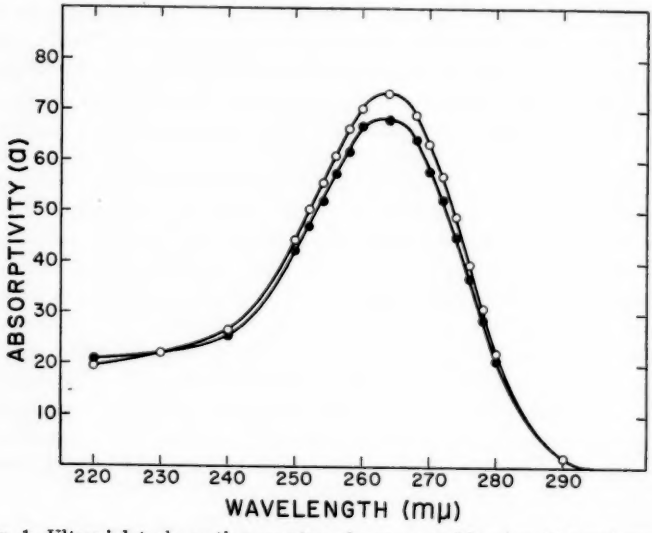

FIG. 1. Ultraviolet absorption spectra of pure ergothioneine (○) and ergothioneine isolated from pig blood (●) in 95 per cent ethanol.

radioactivity of 200 c.p.m. in a windowless flow counter. Additional purification was obtained by chromatography of the sample on alumina (10) and a second precipitation with phosphotungstic acid. The material regenerated from this step was crystallized from aqueous ethanol to yield 3.4 mg. of needles. The identity of the substance was confirmed by the intensity of the color produced in the quantitative diazo test (8) and by ultraviolet absorption (Fig. 1). These criteria indicated the material to be ergothioneine of 93 per cent purity. For the detection of radioactivity the sample was spread in a thin film 18 mm. in diameter and examined in the flow counter. No radioactivity above the background level could be detected, even after several hours of counting.

To obtain a measure of the degree of incorporation of S^{35} into the red cells, the washed erythrocytes from 4 ml. of blood obtained at the time of

sacrifice of the pig were oxidized in a Parr bomb with a perchlorate-peroxide mixture, and the resulting inorganic sulfate was converted to barium sulfate. This was collected on a filter paper disk 17 mm. in diameter and examined in the flow counter. The sample, which weighed 12.9 mg., contained 3400 c.p.m.

Although it was not possible to obtain seminal fluid for the isolation of ergothioneine, as Heath and coworkers had done with the mature pig (2), an examination of the seminal vesicular tissue was carried out. The vesicles weighed 8 gm., and chromatographic analysis (10) of a small sample yielded an ergothioneine content of 49.5 mg. per 100 gm. When 5 gm. of the tissue were analyzed chromatographically, a large ergothioneine peak was present in the column effluent (Fig. 2), but no radioactivity could be detected in the ergothioneine-containing fractions.

Feeding of S^{35}-Methionine to Rat—Since our previous experiment (1) with S^{35}-methionine had been carried out by injection of the compound into a rat, we investigated the possibility that the route of administration might be of importance. For this purpose, 1 mc. of S^{35}-L-methionine (192 mg.) was incorporated into the food (Rockland rat diet) of a 310 gm., Sherman strain male rat, and this was fed in equal daily portions for 4 days. The animal was maintained on the stock diet for an additional 17 days and then was sacrificed. 4.3 ml. of blood were analyzed by chromatography on alumina (10). Alternate 1 ml. samples of the column effluent were analyzed for ergothioneine (Fig. 2), and the remaining fractions were examined in the flow counter for radioactivity. No activity above the background was found in any of the fractions in the ergothioneine range. When the red cells from 1 ml. of blood were examined for S^{35} by the procedure described above, a barium sulfate pad weighing 7.8 mg. and showing radioactivity of 41,600 c.p.m. was obtained.

Effect of Dietary Ergothioneine on Blood Ergothioneine Levels—Experiments were carried out to determine the efficiency with which dietary ergothioneine is incorporated into erythrocytes. The blood ergothioneine levels of six 100 gm., Sherman strain rats were lowered to undetectable values by feeding for 3 to 5 weeks a purified diet containing casein as the source of protein (casein 20 per cent, sucrose 55, hydrogenated vegetable oil 19, and salts 4[1] plus vitamins). Pure ergothioneine was added to the diet of pairs of animals at levels of 0.01, 0.005, or 0.001 per cent. In rats consuming the 0.01 per cent ergothioneine diet the blood values were approximately 4 mg. per 100 ml. in 2 weeks, increasing to 8 mg. per 100 ml. in 4 weeks. The 0.005 per cent diet produced levels of 1.5 and 4 mg. per 100 ml. at 3 and 5 weeks, respectively. On the 0.001 per cent diet levels of 1 mg. per 100 ml. were obtained after 8 weeks of feeding.

The blood levels attained in these experiments are only roughly indicative

[1] Osborne-Mendel.

of the efficiency with which dietary ergothioneine is assimilated. More extensive experiments demonstrated an appreciable variation in the rate and degree of incorporation of ergothioneine into the blood cells; for example, values ranging from 2 to 8 mg. per 100 ml. were observed in twenty rats which had been maintained on the 0.005 per cent ergothioneine diet for 11 weeks. This variability is in contrast to the uniform levels observed by Hunter (5). The variations could not be correlated with food intake

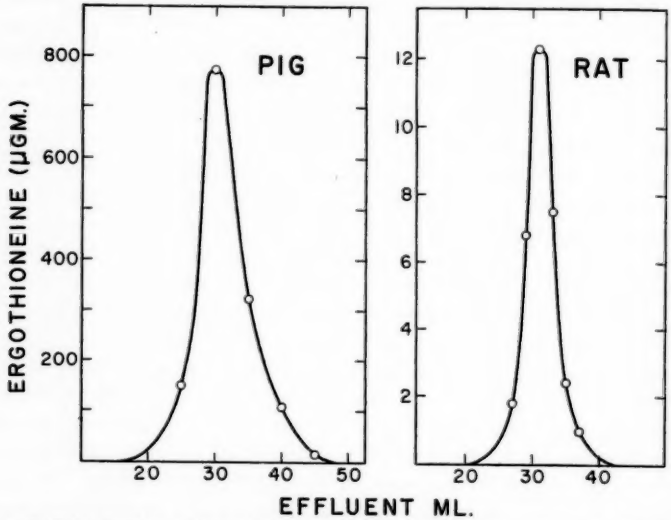

FIG. 2. Distribution of ergothioneine in alumina chromatograms of pig seminal vesicles and rat erythrocytes, from animals which had received 1 mc. of S^{35}-methionine. No radioactivity was detectable in any of the ergothioneine-containing fractions.

or weight gain. As noted below, the nature of the dietary protein also appears to affect the incorporation of ergothioneine into the blood.

Zein Feeding—Because of the suggested precursor effect of the corn protein, zein (3), ten rats were maintained for periods up to 6 months on a purified diet in which casein was replaced by zein fortified with lysine, histidine, tryptophan, threonine, methionine, and valine. No blood ergothioneine was detected in any of the animals. When 0.001 per cent ergothioneine was included in the zein diet of three 100 gm. rats, blood levels of 3 to 5 mg. per 100 ml. were present within 7 weeks, and, when a zein diet containing 0.005 per cent ergothioneine was fed to three other animals, blood levels of 10 to 14 mg. per 100 ml. were observed in 7 weeks.

Precursor Effect of Corn—The purified casein diet was modified by re-

placing part of the sucrose with ground corn (casein 20 per cent, corn 45, sucrose 10). When three 100 gm. rats were fed this corn-casein diet, no blood ergothioneine was detectable at the end of a 12 week feeding period. However, when a corn-zein diet (fortified zein 20 per cent, corn 45, sucrose 10) was fed to two rats, blood levels of approximately 1 mg. per 100 ml. were maintained throughout the 12 week period. Furthermore, it was found that an aqueous acetone extract of corn also permitted maintenance of levels of 1 to 2 mg. per 100 ml. when the extract was incorporated into the 20 per cent zein-55 per cent sucrose diet of two rats in an amount (1.27 gm. per 100 gm. of diet) equivalent to a 45 per cent level of the original corn.

To establish more definitely the precursor effect of corn, seven weanling rats were maintained on the 20 per cent zein-55 per cent sucrose diet for 5 weeks, at which time blood ergothioneine determinations (8) were negative; the rats were then placed on the 20 per cent zein-45 per cent corn diet. Determinations of blood ergothioneine were still negative 2 months later, but after 4 months all the animals showed values of 1 to 2 mg. per 100 ml. To establish beyond doubt that the positive tests were due to ergothioneine, blood samples from two of the animals were analyzed chromatographically (10). In each case a well defined ergothioneine peak was obtained in the column effluent, in an amount equivalent to a blood level of approximately 1 mg. per 100 ml.

DISCUSSION

In the experiment described by Heath and coworkers (2) 1 mc. of S^{35}-methionine was fed to a 220 kilo boar, and seminal fluid was collected on the 39th and 53rd days of the experiment. Crystalline ergothioneine (17.4 mg.) was isolated from the alcohol-soluble fraction of these samples by treatment with phosphotungstic acid. The crystalline material, which did not give a ninhydrin reaction, was further purified by paper chromatography. Ergothioneine was located on the paper by means of the color reaction with 2,6-dichloroquinone chloroimide and was eluted with water. The radioactivity of the eluted material was 37 c.p.m. per sq. cm. at infinite thickness.

The absence of radioactivity in the ergothioneine isolated from pig blood in the present experiment is difficult to reconcile with the results of Heath and coworkers. The smaller size of our animal (weight 30 kilos) permitted attainment of higher levels of radioactivity in the sulfur of the red cells: 3400 c.p.m. in the barium sulfate from 2 ml. of cells, compared with a maximal level of 174 c.p.m. per sq. cm. at infinite thickness in the barium sulfate from 4 ml. of cells obtained by Heath *et al.* 11 and 25 days after the administration of S^{35}-methionine.

It might be argued that blood ergothioneine is of dietary origin, while

ergothioneine from seminal fluid is synthesized by the boar. It has been found by Mann and Leone (11) that the seminal vesicles are the source of seminal fluid ergothioneine in the boar. Although the animal used in the present experiment was not mature enough to provide seminal fluid, it should be noted that the seminal vesicular tissue contained appreciably greater amounts of ergothioneine than did the red cells. While this ergothioneine was not obtained in a pure state, it is significant that no radioactivity was associated with it. It seems not unreasonable to believe that the origin of this ergothioneine is the same as that of ergothioneine secreted by the seminal vesicles of the mature animal. Heath and coworkers (2) have demonstrated that dietary ergothioneine appears in the seminal plasma of the boar.

It is possible that seminal ergothioneine of the mature boar arises both from the diet and from synthesis. However, we should point out that in the isolation of ergothioneine from pig blood a rather lengthy procedure was necessary to remove radioactivity completely from the ergothioneine; a value of 3 to 5 c.p.m. was obtained on the material prior to the final crystallization. The tenacity with which radioactive impurities follow the ergothioneine fractionation might explain the results of Heath *et al.*, inasmuch as no rigid criteria of purity were presented.

The negative results obtained with the rat confirm our previous experiments (1) in which a different route of administration of S^{35}-methionine and smaller doses of radioactivity were employed.

The feeding experiments with corn demonstrate that this grain can serve as a dietary source of ergothioneine for rats, and to that extent they support the conclusion of Eagles and Vars (3) that corn serves as a dietary source of ergothioneine for pigs. In the experiments reported here the precursor effect is slight. However, it seems likely that the effectiveness of corn may vary, depending on the source of the grain. Certainly the experiments of Potter and Franke (4) indicate this to be true in the case of oats, and the geographical variations in blood ergothioneine levels noted by Hunter (5) may well be explained on a similar basis. That other factors, both internal and external, operate to affect blood ergothioneine levels is shown by the different values obtained with fortified zein compared to casein diets, and by the individual variations observed in rats subsisting on identical diets containing known amounts of ergothioneine.

The nature of the substance in corn which gives rise to blood ergothioneine is unknown. It is significant that zein did not raise blood ergothioneine levels, while an aqueous acetone extract of corn did. This suggests that the unknown substance is not associated with the protein fraction of corn. Should the substance be ergothioneine itself, the inability of investigators to detect its presence in corn becomes understandable in the light

of experiments showing that as little as 1 part of ergothioneine in 100,000 parts of diet can give rise to demonstrable blood levels. The high blood ergothioneine reported by Baldridge and Lewis (7) and by Baldridge (13) after feeding oats suggests that ergothioneine might be detected more readily in this grain.

Appreciation is expressed to Dr. Richard E. Shope for supplying the pig used in these studies, and to Miss Mary Exner for carrying out many of the blood ergothioneine determinations.

SUMMARY

1 mc. of S^{35}-methionine was fed to a young pig in an attempt to determine whether or not red cell ergothioneine is synthesized in this species. The crystalline ergothioneine isolated from the blood was devoid of radioactivity. The ergothioneine of the seminal vesicles was likewise inactive. Similarly, no evidence was obtained for the synthesis of blood ergothioneine by the rat after feeding 1 mc. of S^{35}-methionine.

In feeding experiments with rats it was established that corn can serve as a precursor of blood ergothioneine. An aqueous acetone extract of corn produced similar effects. A dietary concentration of ergothioneine as low as 1 part in 100,000 leads to the accumulation of ergothioneine in rat erythrocytes.

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A KINETIC ANALYSIS OF THE GLYCOLYTIC RATE AND CERTAIN GLYCOLYTIC ENZYMES IN NORMAL AND LEUCEMIC LEUCOCYTES*

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It is generally agreed that human leucocytes have high aerobic glycolytic rates (1) and previous studies (2, 3) have shown the rates in homogenates of isolated normal leucocytes to be significantly higher than in leucemic cells. The present studies seek to explain this metabolic difference in terms of possible differences between the enzymic mechanisms of glycolysis in normal and leucemic leucocytes.

Throughout the huge literature on glycolysis, it is often stated that the glycolytic rate of a tissue is determined by a single, rate-limiting step in the sequential chain of reactions although its identity is unknown. Nor is it known whether the rate-limiting step is the same in all tissues or whether it remains the same in pathological variants showing altered glycolytic rates. Some evidence has implicated the cell adenosine triphosphate-adenosine diphosphate ratio (4) as the determinant of glycolytic rate, a ratio at least partially controlled by the balance between adenosinetriphosphatase and hexokinase, but the data are inconclusive. Presumably, that ratio would influence the activity of adenylic acid-linked enzymes, one of which might then become rate-limiting. Burton (5) and Hinshelwood (6) have emphasized the possible hazards of the "master reaction" concept by pointing out that in a sequence of irreversible first order reactions

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

where A, B, and C are the reactants and k_1 and k_2 are the velocity constants of the reactions, the rates of change in the concentrations of the reactants at time t may be expressed by the equations

$$-\frac{dA}{dt} = k_A A, \qquad \frac{dB}{dt} = k_A A - k_B B, \qquad \frac{dC}{dt} = k_B B$$

The rate of production of C thus depends upon $k_A A$ and $k_B B$ and not upon either alone unless one is considerably greater than the other.

* Based on work performed under contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

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An enzyme could function as pace-maker in a multienzyme system for many reasons, among which are spatial location, pH or thermodynamic conditions, low apoenzyme concentration, coenzyme or activator deficiency, low coenzyme or substrate affinity or diffusibility, and presence of inhibitors. Thus, although the *functional capacity, i.e.* $V_{max.}$, of an individually examined enzyme reaction may be demonstrably high, its actual performance as a link in a metabolic chain may be low or limiting for all or any of these reasons.

In a stimulating essay (7), Dixon wrote "it is scarcely practicable to work out the complete kinetics of a chain of enzyme reactions quantitatively" and indeed little has been done along these lines in single tissues and with systematic consideration of the above factors. Leucocytes are well suited for a study of this type because of their high aerobic glycolytic rate, low oxygen consumption, availability in "pure culture," ease of enumeration and morphological study, and variety of pathological analogues. A number of glycolytic enzymes and coenzymes have been studied, and an attempt is made here to compare them in normal and leucemic cells and to correlate their properties with those of the over-all glycolytic process as measured under apparently optimal conditions.

Materials and Methods

Clinical Material—Data were obtained on the leucocytes of forty-eight normal subjects, forty-five patients with chronic myelocytic leucemia, and thirty-five patients with chronic lymphocytic leucemia. The myelocytic leucemia patients had leucocyte counts averaging 103,000 per c.mm. with a range of 66,500 to 330,000. All differential counts showed cellular immaturity, myelocytes, metamyelocytes, and band forms predominating. The average percentage of myeloid cells was 95 per cent. The patients with lymphocytic leucemia had counts ranging from 37,750 to 430,000 per c.mm. with a mean of 123,000 and an average lymphocyte percentage of 97. Although the leucocyte populations of single bloods did contain more than one morphological form, one cell type predominated. Within the three clinical groups studied, leucocyte differential patterns were essentially similar.

Isolation of Leucocytes and Preparation of Homogenates—Details of these procedures have been published previously (2). Special care was used to obtain erythrocyte-free preparations. Prior to homogenization, six cell counts were done on the saline suspensions. The standard deviation of the mean of the suspension counts was computed and expressed as a percentage. The mean standard deviation of a series of 187 suspensions was found to be 8.1 per cent, and the standard deviation of this mean was 2.1 per cent.

Suspensions were homogenized for 4 minutes in ice-cold, glass McShan-

Erway homogenizers (8). Microscopically, at least 80 per cent of the cells appeared to have been disrupted. Nuclei and other subcellular components appeared intact. In testing these homogenates, it was shown that 10 minutes centrifugation at 600 × *g* eliminated nuclei and unbroken cells. The resulting supernatant fraction contained 60 to 80 per cent of total homogenate nitrogen. In some experiments, homogenates were prepared in the Nelco blender and the French pressure cell (9).

Reagents—Stock solutions of ATP[1] (Pabst), DPN (90 per cent, Sigma), cytochrome *c* (Nutritional), and fructose 1,6-diphosphate (Schwarz) were neutralized and kept frozen. Bovine fibrinogen (Armour), glucose (Merck), sodium pyruvate (Eastman), nicotinamide (Eastman), cysteine hydrochloride (Nutritional), and DPNH (90 per cent, Sigma) were freshly prepared. Calcium salts of DHA-P, G-3-P, and α-GP (Delta) were converted to sodium salts with Amberlite IR-120 cation exchange resin. G-3-P dioxane bromide addition compound (Concord) and G-3-P and DHA-P obtained from cyclohexylammonium salts of their dialkyl acetal derivatives (the gifts of Dr. Clinton Ballou) were used in some experiments. Reagent triose phosphates were assayed enzymatically, chromatographically, and by alkali-labile phosphorus determinations.

Aldolase and G-3-PDH were purified from rabbit muscle according to Taylor, Green, and Cori (10) and Cori, Slein, and Cori (11), respectively. Isomerase was purified from rabbit muscle according to Meyerhof and Beck (12). A sample of yeast protein No. 2 (G-3-PDH) was kindly provided by Dr. Edwin Krebs.

Assay Methods—All enzymes were determined at 38°. Aldolase was measured by a modification (13) of the procedure of Sibley and Lehninger (14). Isomerase was assayed by a method developed in this laboratory (15) and the method of Warburg and Christian (16), DPNase by the cyanide procedure of Colowick, Kaplan, and Ciotti (17), and total pyridine nucleotides in TCA extracts by the fluorometric method of Robinson, Levitas, Rosen, and Perlzweig (18), with methylnicotinamide iodide[2] as the reference standard. Ce^{++++} was added to the assay system to oxidize reduced tissue pyridine nucleotides. The results are expressed as micromoles of DPN. Nitrogen was measured by the standard micro-Kjeldahl method. Details of the α-GPDH assay are given in Table II.

LDH was assayed spectrophotometrically by measuring ΔD_{340} at 30

[1] The following abbreviations are used: ATP, adenosine triphosphate; DHA-P, dihydroxyacetone phosphate; DPN, diphosphopyridine nucleotide; DPNase, diphosphopyridine nucleotidase; DPNH, dihydrodiphosphopyridine nucleotide; α-GP, α-glycerophosphate; G-3-P, glyceraldehyde-3-phosphate; α-GPDH, α-glycerophosphate dehydrogenase; G-3-PDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactic dehydrogenase; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; HDP, fructose-1,6-diphosphate.

[2] Kindly prepared by Dr. David Howton.

seconds in the presence of tissue, excess DPNH, and pyruvate after aging the homogenate for 3 to 5 hours at 4° to promote destruction of endogenous DPN, thus eliminating the small ΔD noted in fresh homogenates in the absence of added substrate. Aging in the presence of nicotinamide preserved this endogenous ΔD. The reaction mixture used in the routine assays contained (final concentration in parentheses) DPNH (6.25×10^{-5} M), sodium pyruvate (5.5×10^{-4} M), nicotinamide (0.55 M), phosphate buffer, pH 7.4 (0.033 M), and 0.1 ml. of leucocyte homogenate diluted to contain the equivalent of 5×10^6 cells (added at zero time) per 3 ml. in a 1 cm. silica cell. Data were taken from a Brown recorder attached to a Beckman DU spectrophotometer. In computing results, the ΔD at 60 seconds (2×30 seconds ΔD) was converted to micromoles of DPNH oxidized per ml. per minute per 10^8 cells, with 6.22 as $\epsilon^{1\,\text{cm.}}_{\text{moles}}$ of DPNH (19). The blank contained all additions except DPNH.

G-3-PDH was assayed spectrophotometrically as ΔD_{340} at 30 seconds in a system containing (final concentration in parentheses) fructose 1,6-diphosphate (0.005 M) or G-3-P (0.0005 M), DPN (0.0001 M), cysteine (0.0004 M), potassium arsenate (0.006 M), KF (0.04 M), Tris buffer, pH 8.5 (0.03 M), and 0.1 ml. of "aged" leucocyte homogenate containing 5×10^6 cells per 3 ml. in a 1 cm. silica cell. When fructose 1,6-diphosphate was used as substrate-precursor, homogenate was added to cuvettes containing all constituents except DPN and was incubated for 30 minutes at 38°. The reaction was initiated by adding DPN. The results were calculated as for LDH. When G-3-P was used as substrate, the system was not preincubated.

In determining Michaelis constants for G-3-PDH, two methods were used to vary the concentration of G-3-P. As shown in Fig. 1, *A*, when G-3-P was formed by preincubation of tissue with fructose 1,6-diphosphate, the reciprocal of observed velocity ($1/v$) was a linear function of the reciprocal of duration of preincubation ($1/t$). Since triose phosphate production by tissue aldolase proceeded linearly with or without added hydrazine for 60 minutes (Fig. 1, *B*), the concentration of G-3-P could be varied when fructose 1,6-diphosphate was its precursor by varying the duration of preincubation. The actual concentration of G-3-P at the end of preincubation presumably depended upon the relative activities of tissue aldolase, triosephosphate isomerase, and G-3-PDH and, hence, had to be specifically determined in parallel tubes by colorimetric methods described elsewhere (13). Fig. 1, *C* illustrates an analysis of the G-3-PDH assay system with fructose 1,6-diphosphate as substrate-precursor. When G-3-P was used as substrate, initial concentrations could be varied directly. Fig. 1, *D* illustrates the linear continuity in a Lineweaver-Burk plot of points obtained by both methods of substrate variation with one homogen-

ate. Since calculations of the Michaelis constant for substrate (K_s) for G-3-PDH were based on substrate concentrations at zero time, they would be subject to whatever error may result from G-3-P removal, owing to

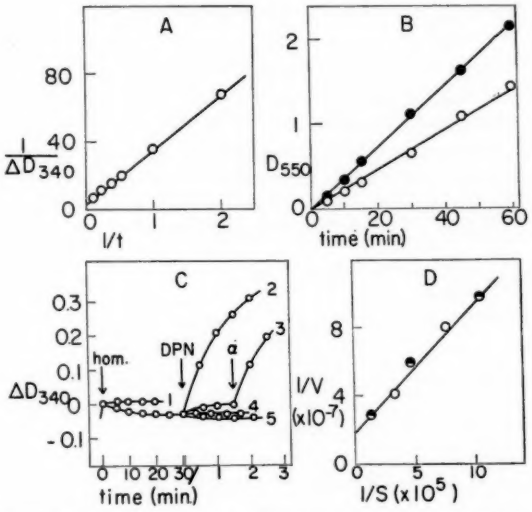

FIG. 1. Methods of varying [s] in the determination of G-3-PDH kinetics. *A*, plot of reciprocal of observed ΔD in G-3-PDH assay with fructose 1,6-diphosphate as substrate-precursor *versus* reciprocal of duration of preincubation period. Details in the text. *B*, time-course of aldolase reaction with hydrazine added (●) and omitted (○). *C*, the G-3-PDH assay with fructose 1,6-diphosphate as substrate-precursor. Time scale of preincubation period is compressed. All cuvettes contained potassium arsenate (0.006 M), KF (0.04 M), and Tris buffer, pH 8.5 (0.03 M), all but those for Curve 3 contained cysteine (0.0004 M), and all but those for Curve 4 contained fructose 1,6-diphosphate (0.0005 M). Curve 1, DPN (0.0001 M) added before homogenate, no preincubation; Curve 2, homogenate added at zero time and preincubated for 30 minutes; reaction initiated with DPN; Curve 3, cysteine omitted from preincubation. DPN promoted no reaction until cysteine added at α; Curve 4, fructose 1,6-diphosphate omitted throughout; Curve 5, hydrazine (0.03 M) added to preincubation mixture. *D*, Lineweaver-Burk plot of K_s for leucocyte G-3-PDH. s = G-3-P (○); substrate-precursor = HDP (◓).

simultaneous isomerase activity, and would tend to be high. The tissue levels and K_s values of isomerase to be reported below suggest that this source of error would not substantially alter the magnitude of the calculated K_s values for G-3-PDH.

Aerobic glycolysis was measured in air in a system containing glucose or fructose 1 6-diphosphate (0.0053 M), phosphate buffer, pH 7.4 (0.01 M),

$MgCl_2$ (0.0053 M), ATP (0.0011 M), DPN (0.007 M), and cytochrome *c* (1.4×10^{-5} M), details concerning which have been published (2). Lactic acid was measured by the method of Barker and Summerson (20). Lactic acid production proceeded linearly for 60 minutes in most experiments. Lactic acid production per minute was calculated from the 20 minute rate.

TABLE I

Comparisons of Normal, Myelocytic Leucemia, and Lymphocytic Leucemia Leucocyte Aerobic Glycolytic Rate, Glycolytic Enzyme Activity, and Pyridine Nucleotides

The figures represent activity levels relative to mean activity in normal cells (100) ± standard error of the mean, except those for pyridine nucleotides which are given in micromoles. Figures in bold-faced type differ significantly from normal ($P < 0.001$).* Number of cases studied in parentheses.

Type of leucocyte	Aerobic glycolytic rate	G-3-PDH	LDH	Aldolase	Isomerase	Pyridine nucleotides
			Data calculated per 10^{10} cells			
Normal	100 ± 12 (13)	100 ± 12 (9)	100 ± 12 (13)	100 ± 6 (18)	100 ± 11 (13)	1.39 ± 0.14 (10)
Myelocytic leucemia	**42 ± 5** (12)	**59 ± 6** (8)	**41 ± 3** (14)	107 ± 5 (12)	106 ± 8 (5)	1.26 ± 0.13 (11)
Lymphocytic leucemia	**16 ± 2** (10)	**19 ± 2** (6)	**24 ± 3** (10)	**35 ± 1** (12)	**32 ± 3** (4)	1.27 ± 0.13 (8)
			Data calculated per mg. tissue nitrogen			
Normal	100 ± 12	100 ± 12	100 ± 12	100 ± 6	100 ± 11	0.77 ± 0.08
Myelocytic leucemia	**45 ± 5**	**67 ± 7**	**43 ± 3**	115 ± 5	113 ± 8	0.75 ± 0.08
Lymphocytic leucemia	**40 ± 5**	**46 ± 5**	**59 ± 7**	86 ± 3	80 ± 6	1.75 ± 0.18

* Calculation of standard error of the mean, s.e. $= \sqrt{\Sigma d^2/(n(n-1))}$. P derived by Fisher's "t" test.

EXPERIMENTAL

Enzyme and Pyridine Nucleotide Levels—Table I presents the mean levels of glycolytic activity, enzyme activity, and pyridine nucleotide concentration found in a survey of normal, myelocytic leucemia, and lymphocytic leucemia leucocyte homogenates. Because measurements of glycolysis and enzyme activity were performed under arbitrary conditions, the data are meaningful chiefly as they permit comparison of the three tissues. The results, therefore, are presented on the basis that activity in normal cells equals 100, except for pyridine nucleotide levels which are

given in micromoles. Data are shown both as calculated per cell and per mg. of N to dispel the possible influence of cell size on activity ratios.[3]

It is seen that the observed levels of dehydrogenase activity closely paralleled glycolytic activity, whereas aldolase and triosephosphate isomerase activity were, if anything, higher in myelocytic leucemia leucocytes, while glycolysis was lower than normal. These conclusions are not substantially altered when data are calculated per mg. of N, although the patterns in the two leucemic tissues are brought closer together by this calculation. Pyridine nucleotides were about the same per cell but higher per mg. of N in lymphocytic leucemia leucocytes.

Several comments are in order concerning the assay methods. Because, in the G-3-PDH assay, substrate was produced from fructose 1,6-diphosphate by endogenous tissue aldolase, DHA-P accumulated along with the G-3-P, presumably exceeding it in concentration owing to triosephosphate isomerase activity. Tissue α-GPDH, if present, would reduce DHA-P and oxidize DPNH simultaneously with G-3-PDH-catalyzed DPN reduction, thus lowering the observed ΔD (21). To assess the extent of possible interference from this source, the tissue α-GPDH reaction was measured in both directions in systems containing DHA-P plus DPNH and α-GP plus DPN. Activities were compared with ΔD values obtained in the G-3-PDH assay with G-3-P as substrate and identical quantities of homogenate. As shown in Table II, α-GPDH activity was low in the tissues studied.

Since aldolase activity was demonstrably low in lymphocytic leucemia cells, G-3-P (5×10^{-4} M) was used as substrate in surveying lymphocytic leucemia leucocyte G-3-PDH activity to permit comparison of the three tissues at essentially equal substrate concentrations.

The demonstration that in brain measurable DPNase is related to particle size following homogenization (22) suggested a possible source of error in the pyridine nucleotide method. This was investigated with four degrees of particle size: intact cells, McShan-Erway glass homogenizer, Nelco blender, and French pressure cell homogenate with and without added nicotinamide. Particle size was roughly gaged by microscopic inspection and by measuring the per cent of total homogenate N in the supernatant fraction after centrifugation of the whole homogenate for 10 minutes at $600 \times g$. Table III presents the effect of added nicotinamide and particle size on measurable DPNase activity and fluorometrically meas-

[3] A large series of determinations of normal, myelocytic leucemia, and lymphocytic leucemia leucocyte homogenate total nitrogen gave mean values (± standard error) of 180.2 ± 11.4, 168.6 ± 11.6, and 72.2 ± 1.8 mg. of N per 10^{10} cells, respectively, reflecting the morphologically obvious size difference between granulocytes and lymphocytes.

ured pyridine nucleotide. Unless nicotinamide was added, the demonstrable pyridine nucleotide level was critically dependent on the degree of

TABLE II

Relative Activities of α-GPDH and G-3-PDH in Leucocyte Homogenates As Determined in Systems Containing Pure Substrates

The figures represent observed ΔD_{340} per 30 seconds per 10^7 cells. α-GPDH was measured spectrophotometrically in two systems: (*a*) *s* = α-GP and *c* = DPN and (*b*) *s* = DHA-P and *c* = DPNH. The cuvettes contained (final concentration in parentheses) (*a*) α-GP (0.006 M), DPN (0.003 M), phosphate buffer, pH 7.4 (0.01 M), "aged" leucocyte homogenate (5×10^6 cells), and water to 3.0 ml. and (*b*) DHA-P (0.006 M), DPNH (0.003 M), and buffer, water, and homogenate as above. Because α-GPDH reactions were initiated with homogenate, separate tubes containing no added substrate or coenzyme were run to control endogenous optical activity. G-3-PDH was assayed as described in the text, with G-3-P as substrate.

Type of leucocyte	G-3-PDH	α-GPDH		Endogenous	Calculated α-GPDH	
	s = G-3-P; *c* = DPN	*s* = DHA-P; *c* = DPNH	*s* = α-GP; *c* = DPN	*s* = 0; *c* = 0	*s* = DHA-P	*s* = α-GP
Normal	+0.145	−0.038	−0.020	−0.024	−0.014	+0.004
Myelocytic leucemia	+0.105	−0.028	−0.013	−0.016	−0.012	+0.003
Lymphocytic leucemia	+0.033	−0.012	−0.008	−0.006	−0.006	0

TABLE III

Effect of Degree of Homogenization and Addition of Nicotinamide on Measurable DPNase Activity, Fluorometrically Determined Pyridine Nucleotides, and Supernatant Nitrogen in Myelocytic Leucemia Leucocytes

All figures are percentage values. For pyridine nucleotides and DPNase, the maximal levels found in pressure cell homogenates are designated 100 per cent. When used in pyridine nucleotide assays, nicotinamide, 0.1 M, was added to the cell suspension before homogenization. For nitrogen, the figures represent *actual* per cent of total homogenate nitrogen in supernatant fraction after 10 minutes centrifugation of whole homogenate at $600 \times g$.

Preparation	Supernatant nitrogen	DPNase	Pyridine nucleotides	
			− Nicotinamide	+ Nicotinamide
Intact cells	9.6	22.5	22.4	65.4
All-glass homogenate*	67.0	37.2	75.2	87.5
Nelco blender homogenate	79.0	63.0	29.9	93.1
French pressure cell homogenate	83.2	100.0	10.0	100.0

* Prepared by grinding for 4 minutes in all-glass McShan-Erway homogenizer.

pyridine nucleotide and DPNase liberation and was maximal in McShan-Erway homogenates. Pyridine nucleotide values in McShan-Erway

homogenates, with nicotinamide present, closely approached maximal values and this technique was used for routine assays.

Comparison of Normal and Leucemic Enzymes—The observed differences in the activity levels of individual enzymes among the three tissues raises the following question: are the differences attributable to quantitative differences in the amount of enzyme activity per cell or to qualitative differences in normal and leucemic cell enzymes? In an attempt to answer this question, Michaelis constants were determined for the individual enzymes and for glycolysis (in the fortified system with glucose as substrate). The results as derived from Lineweaver-Burk plots are presented in Table IV.

TABLE IV

K_s and K_c for Glycolysis and Glycolytic Enzymes

For "two substrate" systems in which [c] or [s] is fixed, the concentration of fixed substrate is given. All values are in moles per liter.

Type of leucocyte	K_s				
	Glycolysis	G-3-PDH	LDH	Aldolase	Isomerase
	s = Glucose	s = G-3-P; $c = 1 \times 10^{-4}$ M	s = pyruvate; $c = 6.3 \times 10^{-5}$ M	s = HDP	s = G-3-P
Normal	2.0×10^{-4}	4.0×10^{-5}	4.8×10^{-5}	7×10^{-4}	4.0×10^{-4}
Myelocytic leucemia	3.2×10^{-4}	4.4×10^{-5}	5.0×10^{-5}	7×10^{-4}	3.5×10^{-4}
Lymphocytic leucemia	2.2×10^{-4}	3.8×10^{-5}	5.0×10^{-5}	8×10^{-4}	3.8×10^{-4}
	K_c				
	c = DPN	c = DPN; $s = 0.5 \times 10^{-3}$ M	c = DPNH $s = 0.5 \times 10^{-3}$ M		
Normal	*	4.0×10^{-5}	2.4×10^{-5}		
Myelocytic leucemia	*	2.9×10^{-5}	2.5×10^{-5}		
Lymphocytic leucemia	*	3.4×10^{-5}	2.7×10^{-5}		

* See the text.

K_s values of the four enzymes differed considerably, but the constants for the three tissues were similar. K_s for glycolysis was measured by determining the effect of systematic variation in glucose concentration (s) on lactic acid production per minute (v). Plots of v *versus* s gave rectangular hyperbolae and their reciprocals, $1/v$ *versus* $1/s$, gave straight lines. In the absence of added glucose, lactic acid production was almost negligible

(<10 per cent $V_{max.}$); hence in the derivations v can be related almost entirely to added s. It is interesting that the observed K_s for glycolysis is somewhat higher than that reported for purified brain hexokinase (23).

However, in attempting to measure the DPN concentration at which glycolysis was $\frac{1}{2}V_{max.}$, it was found that omission of added DPN depressed glycolysis in myelocytic leucemia and lymphocytic leucemia leucocytes to only 50 to 60 per cent of $V_{max.}$ and in normal leucocytes to only 90 per cent of $V_{max.}$ (3), as might be expected in whole homogenates in which reversible coupling of the DPN-DPNH system permits catalytic rather than stoichiometric coenzyme concentrations. DPN can be considered a "second substrate" only with pure enzymes or in homogenates when reverse reactions have been eliminated or controlled. If it is assumed that the pyridine nucleotides, as measured above, are dispersed throughout the cytoplasm only (neglecting for this calculation the effect of DPN binding), that cell pyridine nucleotide is 90 per cent DPN, that the cytoplasm represents 65 per cent of the cell volume, and that 10^{10} normal leucocytes occupy 6.0 ml., calculation shows that DPN in normal leucocyte cytoplasm is 3.2×10^{-4} M or 13 times greater than the observed Michaelis constant for DPN (K_c) for LDH, the latter value having been determined in homogenate preparations substantially freed of endogenous DPN and, as noted below, in purified preparations of leucocyte LDH. The increase in glycolysis which follows addition of DPN may therefore reflect the extent of enzymic DPN destruction.

Fig. 2 shows typical Lineweaver-Burk plots of normal, myelocytic leucemia, and lymphocytic leucemia leucocyte LDH activity with varying DPNH levels at two fixed substrate concentrations. Although derived K_c values were identical for the three tissues, calculated $V_{max.}$ values were linear functions of cell LDH concentration, as shown in Fig. 2, *D*, where $V_{max.}$ is plotted *versus* the mean of LDH levels observed in the above survey.

Because Michaelis constants were determined with whole homogenates, the possibility remains that variations in observed enzyme velocity produced by varying substrate or coenzyme concentration resulted indirectly from varying levels of activity in other enzyme systems. To resolve this question for LDH, highly purified enzyme preparations were isolated from normal, myelocytic leucemia, and lymphocytic leucemia leucocytes by a modification of the procedure of Gibson, Davisson, and Bachhawat (24). In a typical preparation (3.68 ± 0.11) $\times 10^{10}$, washed, intact leucocytes (approximately 23 ml. of packed wet cells) were subjected to homogenization for 5 minutes in the Nelco blender in ice-cold 0.154 M KCl solution. The material was centrifuged at $800 \times g$ at 0° for 30 minutes. The supernatant fraction was rehomogenized and centrifuged twice more. The pooled supernatant solutions were adjusted to pH 6.5 with Na_2HPO_4,

0.1 M, and made up to 16 per cent ethanol at −5°. Following centrifugation at 800 × *g* at −5°, the supernatant solution was dialyzed against 0.5 M NaCl at 0°. Solid ammonium sulfate was added to a concentration of 0.3 gm. per ml. The precipitate was dissolved in distilled water, dialyzed against 0.03 M NaCl at 0°, and, after adjustment to pH 5.8 with acetic acid, brought to 5 per cent ethanol at −3° and adjusted to Γ/2 = 0.03

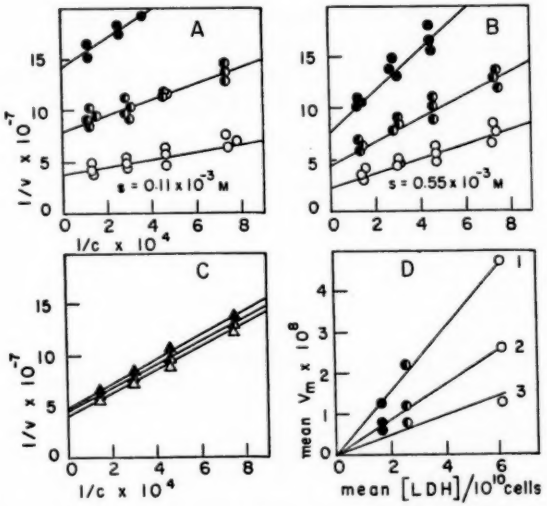

FIG. 2. The kinetics of leucocyte LDH. ○ normal, ◐ myelocytic leucemia, and ● lymphocytic leucemia leucocyte whole homogenates; △ normal, ▲ myelocytic leucemia, and ▲ lymphocytic leucemia leucocyte purified LDH preparations. *A*, Lineweaver-Burk plots of $1/v$ *versus* $1/c$ with $[s] = 0.00011$ M. *B*, same but with $[s] = 0.00055$ M. *C*, same as *B* but with purified enzyme preparations diluted to give identical velocities in the standard LDH system. *D*, abscissa, mean of LDH levels found in survey of clinical groups; ordinate, mean $V_{max.}$ values (× 10^8 calculated from *B*. All graphs were computed by calculations by least squares.

with phosphate buffer, pH 5.8. The clear supernatant fraction obtained on centrifugation was made up to 30 per cent ethanol at −3° and recentrifuged, and the precipitate was redissolved in phosphate buffer, Γ/2 = 0.05, pH 7.7. Reprecipitation at −8° with 30 per cent ethanol was repeated several times and the final residue was dissolved in 1.0 M ammonium sulfate made up in phosphate buffer, 0.1 M, pH 7.8. This product was used for kinetic studies.

Table V shows recoveries and purifications obtained in the preparation described.

As shown in Fig. 2, *C*, kinetic studies on LDH purified from normal, myelocytic leucemia, and lymphocytic leucemia leucocytes yielded identical $V_{max.}$ values. K_c and K_s values were the same as those obtained on whole homogenates. Other leucocyte enzymes have not yet been similarly purified.

The measurement of K_s for LDH (in whole homogenate and purified enzyme) is presented in Fig. 3. Enzyme activity was inhibited at high pyruvate concentrations,[4] and intercepts were obtained by extrapolating the linear segment of the $1/v$ *versus* $1/s$ curves. The mechanism of the substrate inhibition and its possible significance for the calculated Michaelis constants were not investigated further.

TABLE V

Purification of Leucocyte Lactic Dehydrogenase

Fraction	Total nitrogen	Lactic dehydrogenase activity*		
		Total recovery	Per cent recovery	Specific activity
	mg.	*units*		*units per mg. $N_2 \times 10$*
Supernatant of homogenate	529	105	100	2.0
16% ethanol supernatant	235	75	71	3.2
Ammonium sulfate residue	73	75	71	10.3
1st 30% ethanol ppt.	18	37	35	20.6
2nd 30% " "	11	32	30	29.1
Final ammonium sulfate residue	7	30	29	43.0

* 1 unit equals 1×10^{-4} M DPNH converted per minute per ml.

The pH-activity curves obtained for crude and pure LDH, G-3-PDH, and aldolase (Fig. 4) also show functional similarity between normal and leucemic enzymes. The reported specific effect of the buffer used (26) on aldolase was generally confirmed.

The kinetic data permit comparisons of the three tissues in terms of the *maximal velocity* of the individual enzymes and glycolytic rate rather than in terms of the arbitrary activity levels presented in Table I. Here can be compared not only normal tissue with leucemic, but also, and of perhaps equal interest, *absolute capacities* of component glycolytic enzymes with tissue capacity for glycolysis itself. Table VI presents calculated $V_{max.}$ values expressed as micromoles of triose produced per minute per 10^{10} cells.

Despite the apparent proportionality between dehydrogenase activity and glycolytic activity, the capacity for activity of the dehydrogenases

[4] The pyruvate concentration used by Meister (25) in his survey of normal and tumor tissue LDH (3.3×10^{-3} M) was inhibitory for leucocyte LDH.

far exceeds the actual activity which their rôle in glycolysis would seem to demand of them. Aldolase $V_{max.}$ also exceeds that of glycolysis but by a

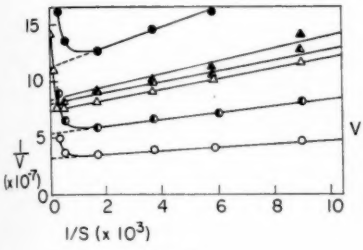

FIG. 3

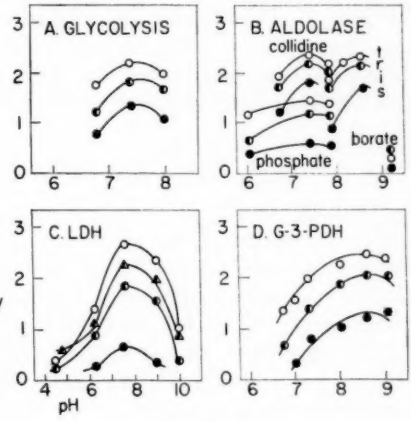

FIG. 4

FIG. 3. The kinetics of leucocyte LDH (continued). Lineweaver-Burk plots of $1/v$ *versus* $1/s$ with $[c] = 6.25 \times 10^{-4}$ M. Symbols defined in Fig. 2.

FIG. 4. pH-activity curves for leucocyte glycolysis, aldolase, LDH, and G-3-PDH. ○ indicates normal, ◐ myelocytic leucemia, ● lymphocytic leucemia leucocyte homogenate, and ▲ LDH purified from myelocytic leucemia leucocytes. Abscissae, pH; ordinates, arbitrary velocity units. Details of incubations in the text. *A*, glycolytic activity. Phosphate buffer, pH 6.8 and 7.4 (0.01 M); Tris buffer, pH 8.0 (0.01 M), with supplemental orthophosphate added (0.01 M). *B*, aldolase. Activities at various pH levels with collidine, Tris, phosphate, and borate buffers. All buffers 0.01 M. Same aliquot of homogenate in each test. *C*, LDH activity. Acetate buffer, pH 4.4; phosphate buffer, pH 6.2 and 7.4; Tris buffer, pH 9.0; and borate buffer, pH 10.2. All buffers 0.033 M. *D*, G-3-PDH. Phosphate buffer, pH 6.8, 7.0, and 7.4; Tris buffer, pH 8.0, 8.6, and 9.0. All buffers 0.03 M.

TABLE VI

Computed Maximal Velocities

Expressed as micromoles of triose produced or converted per minute per 10^{10} cells.

Type of leucocyte	Glycolysis	Aldolase	Isomerase	G-3-PDH	LDH
Normal	75	155	292	512	600
Myelocytic leucemia	33	167	310	320	360
Lymphocytic leucemia	11	53	93	96	180

considerably smaller margin. The similarity between the $V_{max.}$ of LDH and G-3-PDH is also noteworthy.

The results permit construction of the families of curves presented in Fig. 5 where enzyme velocity and glycolysis velocity are plotted against the logarithm of substrate concentration. It should be noted that G-3-PDH and aldolase were measured at pH 8.6 rather than at pH 7.4. The pH-activity curves (Fig. 4) suggest that the comparisons of Fig. 5 would not be substantially altered had all assays been done at pH 7.4. As shown in Fig. 4, *B*, aldolase activity at pH 8.6 with Tris buffer was identical to

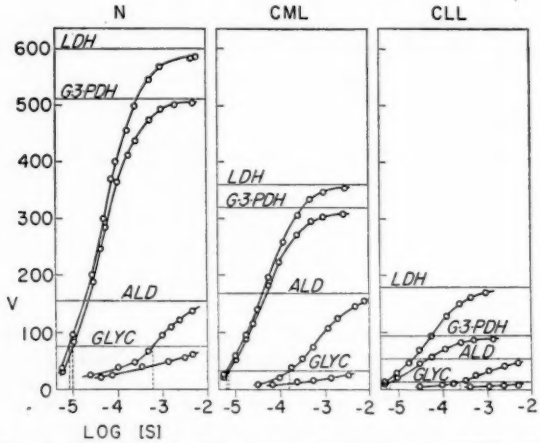

FIG. 5. Graphs showing velocity of glycolytic enzymes (LDH, G-3-PDH, and aldolase) and of glycolysis *versus* logarithm of their substrate concentrations in normal, myelocytic leucemia, and lymphocytic leucemia leucocyte homogenates. Velocities expressed as micromoles of triose formed per minute per 10^{10} cells. The horizontal lines indicate calculated $V_{max.}$ values for glycolysis and designated enzymes. The points where enzyme curves intersect the glycolytic $V_{max.}$ line are projected to the abscissa to indicate substrate concentrations at which individual enzyme velocities would equal glycolytic $V_{max.}$.

that at pH 7.4 with collidine buffer. *In vivo* pH conditions are, of course, not known.

Fig. 5 suggests that, if glycolysis were proceeding at its maximal velocity, the velocities of the component enzymes would necessarily be the ordinate values of the points where their curves intersect the glycolytic $V_{max.}$ asymptote. These points projected to the abscissa imply particular concentrations for the various enzyme substrates at which these velocities will prevail, low in the case of LDH substrate (pyruvate) and G-3-PDH substrate (G-3-P) and higher in the case of aldolase substrate (fructose 1,6-diphosphate). This deduction coincides with the known scarcity of pyruvate and triose phosphate in tissue extracts (27, 28). Fig. 5 also

shows that the somewhat higher than normal $V_{max.}$ of aldolase in myelocytic leucemia leucocytes implies a lower than normal accumulation of fructose 1,6-diphosphate.

DISCUSSION

These conclusions may be considered in the light of Hinshelwood's analysis (6) of steady state metabolism which shows that in a multienzyme sequence

$$k_\mu f_\mu(c_{\mu-1}) = k_{\mu-1}$$

where k_μ and $k_{\mu-1}$ = the velocity constant of the μth and $(\mu-1)$th enzyme of the series, respectively, f_μ = the degree of substrate saturation of the μth enzyme, and $c_{\mu-1}$ = the concentration of substrate provided by the previous enzyme of the series. The steady state could be attained in two cases: (1) with enzyme saturated with substrate, *i.e.* $f_\mu = 1$, k_μ would have to equal $k_{\mu-1}$ and (2) with the enzyme not saturated, its rate being responsive to change in $c_{\mu-1}$, then $c_{\mu-1}$ must adjust itself to such a value that the steady state is established. Were the rate-limiting enzyme for glycolysis shown in Fig. 5, its curve would presumably overlie the glycolysis curve, implying that, at maximal velocity, the enzyme would be substrate-saturated as in the first case of Hinshelwood, while, at submaximal velocities, it would be unsaturated as in the second case. Which of the two cases obtained would therefore depend upon substrate availability. In the absence of "branching" in the sequence, it would appear from the curves that, in steady state metabolism, the non-rate-limiting enzymes (with the possible exception of the first of the sequence) could not be substrate-saturated.

The possible significance of enzymic structural orientation remains undecided in the present case, particularly since the preparations used presumably lacked intact cell surfaces. It has been assumed for the present study that the glycolytic enzymes were uniformly dispersed throughout the cytoplasm (29) and hence the incubation medium. Preliminary experiments have shown that, when ordinary homogenates were centrifuged for 30 minutes at 18,000 × g to eliminate mitochondria and nuclei, the glycolytic rate per unit volume of "remaining supernatant fraction" was about nine-tenths that of whole homogenate levels. Supernatant aldolase was slightly lower. The depressant effect upon glycolysis of omitting added DPN was sizably greater with "remaining supernatant fractions" than with whole homogenate, indicating that DPN loss had accompanied large granule sedimentation.

The assumption that the glycolytic enzymes are soluble in the medium will presumably be supported or refuted by studies now in progress on the

concentrations and turnover rates of glycolytic intermediates. As suggested above, these patterns should be predictable if homogeneous enzyme solutions are involved, diluted though they may be by homogenate technique.

The present data show interesting disparities (1) between normal and leucemic leucocytes and (2) between the relative levels of a multienzyme system and its component enzymes in analogous tissues. Despite their lower than normal glycolytic rate, myelocytic leucemia leucocytes had a normal or higher than normal aldolase content, possibly signifying an additional or different rôle in this tissue for aldolase, an enzyme noted for its lack of absolute substrate specificity. If the lower glycolytic activity in myelocytic leucemia cells were due merely to a smaller cytoplasmic volume, the disparity between aldolase and glycolysis would be relatively even greater.

No differences were demonstrated between the corresponding enzymes of normal and leucemic leucocytes with regard to Michaelis constants and pH-activity relationships, suggesting that the analogous enzymes may be functionally identical. Michaelis constants and pH optima found for leucocyte enzymes substantially agreed with those reported for enzymes from other biological sources (30). K_s for triosephosphate isomerase has not been previously reported.

Fig. 5 suggests that, in the steady equilibrium state, the glycolyzing leucocyte is using at least two of its enzymes well below their maximal capacity, even though their levels are proportional to the glycolytic rate. Of these two, G-3-PDH appears to be the key DPN-reducing enzyme and LDH the key DPNH-oxidizing enzyme. Unlike the situation reported to occur in yeast and muscle, α-GPDH appears to have a minor rôle in the leucocyte "DPN cycle." Chance's analysis (31) of yeast suggests a DPN-DPNH shuttle chiefly between G-3-PDH and α-GPDH. The low levels of α-GPDH found in leucocytes suggest that the G-3-PDH-LDH shuttle is the more significant in this tissue, although the techniques of Chance would be helpful in clarifying the point. It is also interesting in view of the low α-GPDH levels observed to note that triosephosphate isomerase levels and maximal capacities are lower in comparison to aldolase than Meyerhof and Beck (12) reported for other tissues.

The present data reveal no enzyme which can be considered rate-limiting. Experiments to be published later have shown no increase in glycolysis following addition of purified aldolase, isomerase, or LDH to leucocyte homogenate.

Further studies are now in progress on the kinetics of other glycolytic enzymes, particularly hexokinase, phosphofructokinase, and phosphohexose isomerase. Following characterization of the individual enzymes,

it should be possible to examine two, three, and four enzyme subsequences and their relationship to the total sequence. The previously reported (3) increase in glucose glycolysis which follows addition of fructose 1,6-diphosphate suggests replacement of a rate-limiting reaction in the glucose → fructose 1,6-diphosphate subsequence by one in the fructose 1,6-diphosphate → lactic acid subsequence, which may or may not be linked to the adenylic acid system.

SUMMARY

Previously reported differences in aerobic glycolytic rate between normal, chronic myelocytic leucemia, and chronic lymphocytic leucemia leucocytes have been investigated by comparing the activity levels, pH-activity curves, Michaelis constants, and maximal velocities of a number of individual enzymes of glycolysis in the three tissues. The results were compared with similar data on the glycolytic process itself in an attempt to discover the locus of the rate-limiting reaction of the glycolytic sequence.

It was observed that, although the $V_{max.}$ per unit of tissue of any one enzyme studied was higher than the $V_{max.}$ of glycolysis (*i.e.* the rate-limiting enzyme was not found), the activity levels of lactic dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were closely proportional to the glycolytic rate in the three tissues while greatly exceeding it in maximal velocity. Conversely, aldolase and triosephosphate isomerase activity was higher than normal in myelocytic leucemia leucocytes, although the glycolytic rate was lower than normal.

Michaelis constants for substrate and coenzyme and pH optima were essentially identical for the corresponding enzymes of the three tissues, although differing among different enzymes, suggesting functional similarity between normal and leucemic enzymes. α-Glycerophosphate dehydrogenase activity was low and it is suggested that the major DPN-DPNH shuttle in leucocytes occurs between glyceraldehyde-3-phosphate dehydrogenase and lactic dehydrogenase.

Kinetic studies suggest a basis for predicting the concentrations of glycolytic intermediates, if certain presuppositions are valid.

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THE DETERMINATION OF TOTAL BASE OF URINE WITH AN ION EXCHANGE RESIN CONDITIONED AS IODATE*

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The present methods for determining the total base of a urine sample require either considerable time or special apparatus. For purposes of this paper we define the total base of urine as the total base present before ashing and the total fixed base as the total base content after ashing. Total fixed base plus ammonia should approximate the total base, since ammonia is the chief volatile base present in urine. If the method of Vanatta and Cushing (1) is used for the determination of total fixed base by ashing urine in place of serum, low results are obtained because of the high concentration of phosphates in urine. The phosphates form insoluble salts with calcium and magnesium at the high temperature of the ashing. The total base may be determined satisfactorily by the method of Vanatta and Cushing in which no ashing is required.

The chief aim of this work has been to develop a method for the determination of the total base and the total fixed base of urine samples. In determining the total fixed base, the phosphates are eliminated by precipitation with ferric alum (ferric ammonium sulfate) and dry ashing. The excess ferric alum is also removed by precipitation. This procedure is described by Stadie and Ross (2). Ammonia was determined by an aeration method (3).

Apparatus—The apparatus used in the determination is the same as that used by Vanatta and Cushing (1) plus the following: a 25 cc. volumetric flask or test-tube with a 25 cc. calibration mark.

Reagents—The reagents are the same as in the total base determination of serum (1) plus the following:

1. 100 cc. of toluene with two or three crystals (4 mg.) of thymol dissolved in it.

2. 0.1 N ferric ammonium sulfate solution. 15.91 gm. of $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ dissolved in 1 liter of water, which has been conditioned by passing through ion exchange resins. Crystalline ferric alum is stored at 2-4° to prevent decomposition. The solution is stable at room temperature for at least 3 months.

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3. Approximately 8 N solution of NH_4OH. A 1:2 dilution of concentrated NH_4OH is satisfactory.

4. Phenol red indicator solution. 100 mg. in 100 cc. of 95 per cent ethyl alcohol.

Procedure

Titrimetric Determination of Total Fixed Base—Transfer a 5 cc. aliquot of the urine sample to a 25 cc. volumetric flask or to a test-tube with a 25 cc. calibration mark. Add 4 cc. of 4 N H_2SO_4 and 2 drops of phenol red indicator solution. Adjust the solution to pH 6 to 8 by adding 8 N NH_4OH dropwise. Approximately 4 cc. are needed. The indicator changes from a yellow to a red color. 8 to 9 cc. of the 0.1 N ferric alum are added. 8 N NH_4OH is again added dropwise until the dark red color of the indicator is obtained. Approximately 4 drops are required. Dilute the above solution to mark and mix well. Transfer a suitable aliquot to a test-tube and centrifuge for 10 minutes at 2000 r.p.m. Transfer a 5 cc. aliquot of the supernatant solution to a porcelain or Vycor ashing crucible. Add 1 cc. of 4 N H_2SO_4 to the crucible. The sample is then dried, ashed, and carried through the rest of the procedure as described for the ashing of serum. The only modifications necessary are that the volume of phosphotungstic acid and the amount of potassium iodide must be doubled if the total base content exceeds 300 m.eq. per liter.

Titrimetric Determination of Total Base of Urine—Collect the urine sample with preservative, approximately 2 drops per 100 cc. of urine. A standard solution is run through the column of resin in the iodate form and titrated as in the method for serum total base to determine the column error. Transfer 1 cc. of the urine to a 100 cc. volumetric flask and dilute to mark. The remainder of the determination is carried out by the identical procedure of the total base method for serum (1) with the exception that if the total base concentration exceeds 300 m.eq. per liter larger amounts of reagents are needed as described above.

Calculations—These are the same as for the serum total base method (1).

Results

In order to test the stoichiometric reaction of the columns when the ionic concentration varies over a large range, standard solutions of different concentrations were run in a random order. The concentrations used were 30, 45, 75, 105, 150, 225, 300, and 450 m.eq. per liter. The average results of duplicate determinations are given in Table I. The maximal error was −1.5 per cent. For this reason it is felt that the 150 m.eq. per liter standard is adequate to assess column errors regardless of the range of concentrations of urines being analyzed.

To test the effectiveness of the removal of the phosphates by precipitation with ferric alum, a standard solution with total ionic strength of 106 m.eq. per liter containing 60 m.eq. per liter of Na_2HPO_4 was prepared. Sixteen

TABLE I

Errors in Total Base Determination at Different Ionic Strengths As Determined on NaCl Standard Solutions

Theoretical value	Actual value	Per cent error
m.eq. per l.	*m.eq. per l.*	*m.eq. per l.*
30.0	30.3	+1.0
45.0	45.2	+0.4
75.0	74.7	−0.4
105.0	103.9	−1.1
150.0	150.3*	
225.0	223.2	−0.8
300.0	295.6	−1.5
450.0	445.5	−1.0

* Not corrected for column error. Other values were corrected for the difference of this determination from the theoretical.

TABLE II

Averages of Duplicate Determinations of Total Base and Total Fixed Base on Human Urines

The figures in parentheses represent differences of duplicate determinations.

	Sample No.	Total fixed base (*A*)	NH_3 concentration (*B*)	Total base (*C*)	$\frac{A+B-C}{A+B} \times 100$
		m.eq. per l.	*m.eq. per l.*	*m.eq. per l.*	
Normal	1	227.5 (1.1)	97.7	326.6 (0.9)	−0.4
	2	147.9 (0.5)	6.2	154.0 (0.7)	+0.1
	3	76.1 (0.6)	5.0	81.0 (0.3)	+0.1
	4	190.5 (0.5)	53.0	244.1 (1.1)	−0.2
	5	257.1 (0.6)	193.1	452.0 (0.5)	−0.4
	6	220.8 (1.0)	66.5	287.9 (0.4)	−0.2
	7	239.8 (1.0)	71.4	311.0 (0.7)	+0.1
	8	305.3 (1.0)	52.4	358.3 (0.7)	−0.2
	9	148.1 (1.2)	85.3	233.2 (0.7)	+0.1
Hospital	1	163.3 (1.4)	52.7	219.4 (0.5)	−1.6
	2	82.4 (1.0)	47.4	129.3 (0.3)	+0.4
	3	68.3 (0.7)	11.0	77.0 (0.3)	+2.9
	4	45.3 (0.6)	31.4	77.0 (0.2)	−0.4
	5	56.9 (0.4)	71.8	128.0 (2.7)	+0.5
	6	50.0 (0.4)	31.5	81.3 (2.4)	+0.3
Average					+0.07 ±0.24

replicate determinations on this standard solution revealed less than 2 per cent error in all cases.

The total base and total fixed base were determined in duplicate on urines from nine normal persons and six hospital patients. The urines of five of the latter group contained albumin. The accuracy of the results of these determinations is theoretically the same as the accuracy of the standard solutions containing phosphates. The average results of duplicate determinations are presented in Table II. With these values of the total fixed base of the samples, the results of the total base of the urine samples were compared with the total fixed base plus ammonia concentration.

The results of the total base determinations and of the ammonia determinations on the same fifteen samples are reported in Table II. Comparison of values in columns $(A + B)$ and C gives the relative error in the total base, assuming the NH_4OH concentration plus total fixed base concentration to be true total base concentration. The average error here was +0.1 per cent, with a range from −1.6 per cent to + 2.9 per cent.

DISCUSSION

In the determination of the total fixed base of urine by ashing, prior to the removal of the phosphates, the results obtained are low. The phosphates present in urine react with the calcium and magnesium on ashing to form insoluble precipitates which cannot react stoichiometrically on the iodate column. Hald (4) recommends ashing biological material in the presence of ferric sulfate. The phosphates are then theoretically converted to insoluble iron phosphates. This procedure, tried in our laboratory with varying amounts of the ferric sulfate, gave results which ran an average of 8.5 m.eq. per liter low on standard solutions containing 60 m.eq. per liter on dibasic phosphate. Attempts to remove the phosphates with ferric ammonium sulfate proved more successful in our laboratory, as evaluated on standard solutions. Dry ashing is still necessary to remove the excess acids, as they would be converted to HIO_3 on the resin column. It was found that the wet ashing procedure described by Stadie and Ross could be eliminated, even when albumin was present.

An attempt was made to determine total fixed base by the following simplified method: (1) Add to the urine an OH-conditioned resin which has been prepared as described for Reagent 6 by Vanatta and Cox (5) and then dried; (2) remove an aliquot of the alkalized urine and boil to remove the ammonia; (3) dilute 1:100, and continue the determination by passing the diluted solution through the resin column and by completing the titration of an aliquot of the effluent solution. However, we were not satisfied with the results of this because the resin gave either low results if air-dried

or high results if dried at temperatures from 130–150°. The reasons for these difficulties were not apparent. The errors could not be explained by absorption of cations on the resin, nor by a simple decomposition of the resin at these temperatures, in the respective instances.

SUMMARY

Resin-iodate methods for the determination of total base concentration and total fixed base concentration of urine are described. The method for serum total base is modified in order to prevent errors due to the high concentration of phosphates in the urine. The errors of the total fixed base in sixteen replicate determinations on a standard solution were all less than 2 per cent. The total fixed base concentration plus the ammonia concentration was compared with the total base concentration. This comparison showed an average agreement of +0.1 per cent (range −1.6 per cent to +2.9 per cent) on the urines of nine normal persons and on the urines of six hospitalized patients.

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INACTIVATION OF OXIDATIVE AND PHOSPHORYLATIVE SYSTEMS IN MITOCHONDRIA BY PREINCUBATION WITH PHOSPHATE AND OTHER IONS*

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Alteration of mitochondrial integrity or structure by a variety of means has always resulted in a decrease in or elimination of the phosphorylations coupled to electron transfer from pyridine nucleotides to oxygen. Sometimes the oxygen consumption has increased rather than decreased. Perhaps the mildest treatment yet reported to cause marked structural changes in mitochondria is exposure to 10^{-3} M inorganic phosphate. According to Raaflaub (2, 3) mitochondria suspended in isotonic mannitol solution undergo extensive swelling when incubated with this concentration of phosphate.

Because of the possibly critical rôle of structural organization in the coupling of phosphorylation to electron transfer, we undertook a study of the effect of preincubating mitochondria with phosphate on both oxidation and phosphorylation. Although 10^{-3} M inorganic phosphate had no effect under our conditions, preincubation with higher concentrations rapidly inactivated β-hydroxybutyrate oxidation. Further explorations suggested that all DPN-dependent oxidations were affected.[1] Investigation of possible mechanisms revealed that a number of substances produced similar effects and that certain metal ions act in concentrations as low as 10^{-5} M in some preparations. Manganese or magnesium ions, EDTA, and a number of coenzymes protected mitochondria from the detrimental effects of preincubation with phosphate, but could not restore oxidative

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[1] The following abbreviations are used: Tris = tris(hydroxymethyl)aminomethane buffer, pH 7.4; ATP, ADP, and AMP = adenosine tri-, di-, and monophosphates; TPN and DPN = tri- and diphosphopyridine nucleotides; TCA = trichloroacetic acid; PCA = perchloric acid; EDTA = sodium ethylenediaminetetraacetate; GSH = reduced glutathione; BAL = 2,3-dimercaptopropanol; P:O = ratio of micromoles of inorganic phosphate removed to microatoms of oxygen consumed; DNP = 2,4-dinitrophenol.

XUM

activity. DPN, under proper conditions, restored both oxidation and phosphorylation.

Materials and Methods

Reagents—The common chemicals were of analytical reagent grade. The other substances were of the highest purity available. The various coenzymes and nucleotides were obtained from the Sigma Chemical Company or from the Pabst Laboratories. The EDTA was disodium Versenate, reagent grade, Bersworth Chemical Company. Water was redistilled in Pyrex glass. All solutions were adjusted to pH 7.4 with HCl or KOH.

Mitochondrial Preparation—Mitochondria were isolated from rat livers by a slight modification of the method of Schneider (4). The livers were perfused with chilled 0.15 M NaCl and ground with 0.25 M sucrose + 0.02 M Tris buffer, pH 7.4, in a loose fitting stainless steel homogenizer. No effort was made to obtain 100 per cent recovery of mitochondria by washing the sedimented nuclear fraction. The mitochondria were centrifuged at 6000 to 7000 $\times$ g and washed three times with 0.25 M sucrose + 0.02 M Tris. Final suspension in the same medium was made so that 1 ml. was derived from 0.6 gm. of liver.

Preincubation—Substances added during preincubation were dissolved in 0.25 M sucrose, and 0.3 ml. was added to 1.2 ml. of mitochondrial suspension at 0°. In most cases the tubes were incubated at 30° for 10 minutes with occasional shaking, then returned to the ice bath.

Test Incubation—1 ml. of the chilled preincubated suspension was added to chilled Warburg flasks. The medium in the flasks contained the following substances in amounts to give the indicated concentration in the final 2.8 ml. of reaction mixture: 0.013 M potassium phosphate, pH 7.4 (including any phosphate added to the enzyme suspension during preincubation), 0.013 M Tris, pH 7.4, 0.005 M $MgCl_2$, 0.0018 M ATP, 0.3 mg. of 20 per cent pure yeast hexokinase, 0.02 M glucose, 0.02 M NaF, 0.01 to 0.02 M KCl, 0.03 M sucrose, and 0.01 M substrate.

Oxygen consumption was measured at 30° for 30 to 40 minutes. In most experiments the substrate was in the main flask, and the oxygen consumption during the 10 minute equilibration period was considered equal to that in the first 7 minutes following equilibration. When no additions were to be made from the side arm, 0.2 ml. of 30 per cent TCA was placed there and tipped in to terminate the experiment. Otherwise the vessels were chilled and fixed with TCA as quickly as possible. An additional 2 ml. of 10 per cent TCA were added to the flask after removal from the manometer. When analytical procedures involving 260 mμ light absorption were used, 5 per cent PCA was substituted for TCA.

Analyses—Phosphate was estimated by the method of Fiske and Sub-

barow (5). In the presence of arsenate the method of Pett (6) was substituted. AMP, ADP, ATP, and DPN were determined by the adenylic acid deaminase method of Kalckar (7). Changes in turbidity compared to the control mitochondrial suspension in 0.25 M sucrose were rated from 0 to −6, −6 representing an almost clear, though opalescent, solution.

TABLE I

Effect of Preincubation with Phosphate on Oxidation and Phosphorylation with Various Substrates

The mitochondria were suspended in 0.25 M sucrose + 0.02 M Tris in Experiment 1, in 0.02 M Tris in Experiment 2. Preincubations for 10 minutes at 30°; test incubations, 40 minutes. When added, DPN = 1×10^{-3} M, EDTA = 5×10^{-4} M. ΔO_2 is expressed in microatoms.

Experiment No.	1				2					
Preincubation additions	None		0.02 M phosphate		None		0.02 M phosphate			
Turbidity change*	0		−2		0		−2			
Test incubation, special additions									DPN + EDTA	
	ΔO_2	P:O	ΔO_2	P:O	ΔO_2	P:O	ΔO_2	P:O	ΔO_2	P:O
β-Hydroxybutyrate	9.1	2.8	1.4	0	7.2	2.2	0.7	0	7.8	1.4
α-Ketoglutarate	5.6	2.8	0.7	0			0.8	0	7.6	2.0
Succinate	14.8	2.0	16.9	1.4			9.4	0.8	9.9	1.4
Citrate	7.3	2.3	0	0			0.6	0	9.2	1.7
Glutamate	11.1	2.6	0	0			1.0	0	13.1	1.8
Malate							0.5	0	6.0	1.5

* Turbidity decreases were rated from 0 to −6, −6 representing an almost clear but opalescent solution.

Results

Turbidity Changes—In our mitochondrial suspensions, brief preincubation with 10^{-3} M phosphate had no effect, but 0.01 to 0.02 M produced decreases in turbidity which suggested considerable swelling of mitochondria (Table I). Similar decreases in turbidity resulted from preincubation with all other anions and cations which cause inactivation of β-hydroxybutyrate oxidation.

Effect of Preincubation with Phosphate on Oxidation of Various Substrates—When mitochondria suspended in 0.25 M sucrose + 0.02 M Tris were preincubated at 30° for 10 minutes, there was good oxidative and phosphorylative activity with each substrate (Table I). Although α-ketoglutarate oxidation did decline 40 to 50 per cent, β-hydroxybutyrate

oxidation declined very little. When 0.02 M potassium phosphate, pH 7.4, was added to the sucrose-Tris medium, the oxidations involving electron transfer via DPN or TPN were inactivated. Succinate oxidation was slightly increased.

TABLE II

Effect of Preincubation with Various Ions on Oxidation and Phosphorylation

The mitochondria were suspended in 0.25 M sucrose + 0.02 M Tris. Those in Experiments 5 and 7 were from homogenates containing 0.01 M EDTA. Except as indicated the preincubation was always for 10 minutes at 30°. The test incubations were for 30 to 40 minutes. Substrate, β-hydroxybutyrate. ΔO_2 is expressed in microatoms.

Experiment No.	Anions			Experiment No.	Cations		
	Preincubation additions	Test incubation			Preincubation additions	Test incubation	
		ΔO_2	P:O			ΔO_2	P:O
1	None*	7.2	3.0	5	None	5.2	2.6
	Phosphate 0.016 M*	5.9	3.3		$CaCl_2$ 10^{-3} M	0	0
	None	6.2	3.1		" 10^{-5} "	5.0	2.7
	Phosphate 0.016 M	0	0		$ZnSO_4$ 10^{-5} "	0.5	2.3
2	None	6.4	2.7	6	None	6.6	3.1
	Phosphate 0.005 M	5.0	2.5		$ZnSO_4$ 2×10^{-5} M	7.0	2.8
	" 0.01 M	1.2	0		" 6×10^{-5} "	1.3	0
	" 0.02 "	0	0	7	None	8.3	2.7
3	None	7.0	2.9		$CaCl_2$ 10^{-3} M	0.9	0
	NaCl 0.02 M	5.8	3.4		" 10^{-4} "	7.7	2.8
	KCl 0.02 M	6.8	2.8		$BaCl_2$ 10^{-3} "	7.7	2.4
	Phosphate 0.01 M	1.0	0		$SrCl_2$ 10^{-3} "	7.7	2.5
	Arsenate 0.005 "	1.3	0		$CuSO_4$ 10^{-3} "	0	0
	Arsenite 0.002 "	0.3	0		" 10^{-4} "	4.2	0
	Sulfate 0.02 M	5.5	2.8		$FeCl_2$ 10^{-3} "	1.0	1.5
4	None	4.6	2.7		$FeSO_4$ 10^{-4} "	6.8	2.8
	Phosphate 0.02 M	1.3	1.9		$CdSO_4$ 10^{-4} "	0.7	0
	Pyrophosphate 0.02 M	2.0	0.8		$HgCl_2$ 10^{-4} "	0.1	0
	Nitrite 0.01 M	4.3	2.7				
	Nitrate 0.02 "	5.5	2.3				

* Preincubated at 0° instead of 30°.

Inactivation confined to oxidations involving DPN or TPN suggested that the effect of phosphate was in the electron transfer system at the DPN level. Therefore most of the experiments on the mechanism were conducted with β-hydroxybutyrate as substrate. A 10 minute preincubation with 0.02 M phosphate was adopted as a routine procedure, which produced 85 to 95 per cent inactivation. If the preincubation was at 0°, the effect of phosphate was slight (Table II).

With partial inactivation of oxidation, phosphorylation was usually affected to a somewhat greater degree. However, since it has been a common observation that almost any marked inhibition of oxygen consumption is accompanied by some decrease in the P:O ratio, preincubation with phosphate probably has little if any selective effect on phosphorylation. There was active phosphorylation coupled to succinate oxidation, although the P:O ratio was down somewhat from the control value.

Effect of Preincubation with Other Anions—The effect of phosphate during preincubation is not due to simple electrolyte effects, for Tris, NaCl, KCl, $NaNO_2$, $NaNO_3$, and NaF have very little effect (Table II). However, phosphite and pyrophosphate are similar to phosphate in ability to inactivate β-hydroxybutyrate oxidation, arsenate is more active, and arsenite is much more active. In fact, arsenite is the only substance producing rapid inactivation at 0°.

Some variations in effect with different sulfate solutions suggested that impurities might be responsible for the effects seen. Since Slater and Cleland (8) had reported inactivation of heart sarcosomes by Ca^{++}, the effect of EDTA was tested. Addition of EDTA during the preincubation neutralized not only the effect of sulfate but all the effects of phosphate as well (Table IV). Since the concentration of Ca^{++} required (Table II) was considerably greater than could be due to an impurity in reagent quality chemicals, attention was turned to other metal ions.

Effect of Preincubation with Various Metal Ions—In suitable concentrations a number of metal ions produced effects indistinguishable from those of phosphate (Table II). If approximately 90 per cent inactivation of β-hydroxybutyrate oxidation during preincubation is taken as the test criterion, the metal ions tested fall roughly into three groups, as follows: (*a*) metal ions effective at 10^{-5} to 10^{-4} M, Zn^{++}, Cd^{++}, Hg^{++}; (*b*) metal ions effective at 10^{-4} to 10^{-3} M, Ca^{++}, Cu^{++}, Fe^{+++}; (*c*) metal ions having essentially no effect at 1×10^{-3} M, Ba^{++}, Sr^{++}, Cr^{+++}, Co^{++}, Ni^{++}, Mn^{++}.

The experiments with Ca^{++} show results similar to those reported by others (8). It is surprising that Ba^{++} and Sr^{++} had no effect. The minimal concentration of Zn^{++} required for inactivation, as little as 1×10^{-5} M for some mitochondrial preparations, approaches the value which might be present as an impurity in 0.02 M reagent quality Na_2SO_4 or K_2HPO_4. Possibly some untested metal is even more active. Some mitochondrial preparations are much more sensitive to metals than others. Attempts to relate these variations to factors such as the use of EDTA in the homogenizing medium or the amount of contamination with blood, nuclei, or microsomes have been unsuccessful.

Effect of Preincubation with Surface-Active Agents—Since one change produced by preincubating mitochondria with phosphate or metal ions was a decrease in turbidity, suggesting swelling, injury to the mitochondrial

membrane was a possible explanation for the effects. For this reason several surface-active agents were tested (Table III). They all caused inactivation of β-hydroxybutyrate oxidation. The minimal effective concen-

TABLE III

Effect of Preincubation with Surface-Active Agents

The mitochondria were isolated from homogenates containing 0.01 M EDTA and suspended in 0.25 M sucrose + 0.02 M Tris. Preincubation for 10 minutes at 30°; test incubation, 40 minutes. Substrate, β-hydroxybutyrate. ΔO_2 in microatoms.

Experiment No.	Preincubation		Test incubation		
	Additions	Turbidity change	Additions	ΔO_2	P:O
1	None	0		7.8	3.0
	Saponin 0.06%	−2		1.4	0
	" 0.06% + Mn^{++} 5×10^{-4} M	−1		5.2	1.8
2	None	0		6.9	3.2
	Saponin 0.2%	−3		0.9	0
	" 0.2%	−3	DPN 1.3×10^{-3} M	9.6	1.0
	" 0.2% + EDTA 5×10^{-3} M	0		7.7	2.7
	EDTA 5×10^{-3} M	0		5.9	3.4
	Deoxycholate 0.03%	−2		0.2	0
	" 0.03%	−2	DPN 1.3×10^{-3} M	9.1	1.5
	" 0.03% + Mn^{++} 1×10^{-3} M	−1		1.7	0.4
	Deoxycholate 0.03% + EDTA 5×10^{-3} M	−½		5.0	1.9
3	None	0		7.5	2.6
	Lauryl sulfate 0.05%	−5		0.2	0
	" " 0.05%	−5	DPN 0.8×10^{-3} M	8.0	0
	" " 0.05% + Mn^{++} 1×10^{-3} M	0		6.6	0.8
	Lauryl sulfate 0.05% + Mg^{++} 7.5×10^{-3} M	−½		6.3	1.2
	Lauryl sulfate 0.05% + EDTA 1×10^{-2} M	−5		0.5	0

trations (1 or 2×10^{-3} M) produced decreases in turbidity similar to or slightly greater than those seen on preincubation with phosphate or metal ions.

Effect of Preincubation with Uncoupling Agents—Although mitochondria have been reported to undergo structural changes in the presence of DNP because of lack of ATP necessary to maintain their integrity (9), preincubation with 5×10^{-5} M DNP under the conditions reported here re-

sulted in no inactivation of β-hydroxybutyrate oxidation. Gramicidin, 20 γ per ml., more than sufficient for complete uncoupling, caused no change in turbidity or reduction in β-hydroxybutyrate oxidation. Since no attempt was made to wash out either DNP or gramicidin, there was no phosphorylation in the test incubation.

Effect of Suspending Mitochondria in Water—Suspension of mitochondria in water or in 0.02 M Tris yielded preparations with a turbidity as low as or lower than that seen after preincubation with phosphate. These preparations not only retained their full ability to oxidize β-hydroxybutyrate, but also showed phosphorylation nearly equal to that of the controls after preincubation for 20 minutes at 30°. This result was somewhat different from that expected on the basis of the work of Lehninger (10) and Huennekens and Green (11).

Protection against Effects of Preincubation with Various Agents (Tables III and IV)—Protective action was determined by having each substance present during the preincubation period.

EDTA—As expected, this agent neutralized the inactivating effects of metals like Zn^{++}. Completely unexpected, however, was its protective effect against preincubation with phosphate, sulfate, arsenate, arsenite, saponin, and deoxycholate. At the effective protective concentrations, EDTA alone had no effect on oxidation and frequently caused a slight increase in the P:O ratio. Extensive swelling in water and with sodium lauryl sulfate was not prevented by EDTA.

ATP, ADP, AMP—ATP (Table IV) provided fairly complete protection of oxidation and phosphorylation against the inactivating effects of preincubation with phosphate, sulfate, and arsenate. Protection was less against Zn^{++}, and very little against arsenite. ADP was tested only against phosphate, and it proved to be protective. AMP sometimes protected against phosphate and arsenate, but never against arsenite and Zn^{++}. Although Raaflaub (3) reported extremely rapid swelling due to ATP when histidine was present, we have observed no such effect.

Mg^{++}—At 5×10^{-3} M or higher this ion usually gave nearly complete protection against the effects of preincubation with phosphate, sulfate, and arsenate. Against saponin, deoxycholate, and lauryl sulfate there was partial protection of oxidation and smaller but significant preservation of phosphorylation. With arsenite, Ca^{++}, and Zn^{++}, the protective effects of Mg^{++} were slight, but still significant. Simple replacement of lost or diluted Mg^{++} is not the explanation of the effect of Mg^{++} in preventing inactivation, for there was always an ample supply of Mg^{++} in the test incubation medium.

Mn^{++}—This ion is 5 to 10 times more active than Mg^{++} in neutralizing the effect of phosphate during preincubation (Table IV). Although man-

ganese phosphate is a rather insoluble compound, Mn^{++} cannot be acting by removal of phosphate ions, for 5×10^{-4} M Mn^{++} protected against 2×10^{-2} M phosphate. Mn^{++} neutralized the effects of arsenite and of

TABLE IV

Protection against Effects of Preincubation with Various Agents

The mitochondria were suspended in 0.25 M sucrose + 0.02 M Tris. Except in Experiment 2, they were isolated from homogenates containing 0.01 M EDTA. Preincubation for 5 minutes at 30° in Experiments 2 and 3; for 10 minutes in all others. Test incubation for 40 minutes. Substrate, β-hydroxybutyrate. ΔO_2 in microatoms.

Experiment No.	Preincubation: Additions	Preincubation: Turbidity change	Test incubation: ΔO_2	Test incubation: P:O
1	None	0	7.4	2.4
	Phosphate 0.02 M	−2	0.3	0
	" 0.02 " + EDTA 5×10^{-5} M	0	7.0	2.7
2	None		7.9	3.0
	Phosphate 0.016 M		2.0	0
	" 0.016 " + ATP 4×10^{-3} M		7.6	3.0
	" 0.016 " + AMP 2×10^{-3} "		7.6	3.2
	" 0.016 " + Mg^{++} 5×10^{-3} "		6.2	2.7
	" 0.016 " + Mn^{++} 1×10^{-3} "		7.2	2.7
	" 0.016 " + nicotinamide 4×10^{-2} M		5.2	0.5
3	None	0	8.0	2.5
	Arsenate 5×10^{-3} M	−2	1.6	1.4
	" 5×10^{-3} " + AMP 5×10^{-3} M	−1	5.0	1.7
	" 5×10^{-3} " + ATP 5×10^{-3} "	−½	6.9	2.5
	" 5×10^{-3} " + Mg^{++} 7×10^{-3} "	−1	5.5	2.6
	" 5×10^{-3} " + Mn^{++} 1×10^{-3} "	−1	7.3	1.9
4	None	0	6.1	2.7
	Arsenite 2×10^{-3} M	−3	0.1	0
	" 2×10^{-3} " + Mn^{++} 1×10^{-3} M	−½	3.4	2.0
	" 3×10^{-3} " + EDTA 5×10^{-3} "		4.2	2.8
5	None	0	7.6	2.3
	Zn^{++} 3×10^{-5} M	−2	1.0	0
	" 3×10^{-5} " + ATP 5×10^{-3} M	−2	2.8	0.4
	" 3×10^{-5} " + Mg^{++} 7×10^{-3} M	−½	3.0	1.5
	" 3×10^{-5} " + Mn^{++} 5×10^{-4} "	−1	5.7	1.9

Ca^{++} and Zn^{++} better than did Mg^{++}. It also had very significant effects against all three surface-active agents. Although 0.5 to 1.0×10^{-3} M Mn^{++} had little or no effect when added alone to mitochondria, higher concentrations (5×10^{-3} M) actually produced an effect somewhat like that of phosphate.

Other Substances—When DPN or TPN was added with phosphate during the preincubation, there was activity in the subsequent test incubation. Whether this is a true protection or merely restoration of activity by DPN as it is carried over into the test incubation is uncertain. However, a partial protection was observed with nicotinamide (Table IV). BAL had a slight protective effect on oxidation, while GSH, DNP, and NaF had no effect.

TABLE V

Restoration of Oxidation and Phosphorylation after Preincubation with Various Agents

The mitochondria were suspended in 0.25 M sucrose + 0.02 M Tris. Except in Experiments 3 and 4, they were isolated from homogenates containing 0.01 M EDTA. In Experiment 4, the preincubation was for 5 minutes and the test incubation for 30 minutes; in all others the times were 10 minutes and 40 minutes. Substrate, β-hydroxybutyrate. When added in the test incubation, DPN, TPN, and EDTA were 1×10^{-3} M; Mn^{++} was 3.5×10^{-5} M. ΔO_2 in microatoms.

Experiment No.	Preincubation		Test incubation		
	Additions	Turbidity change	Additions	ΔO_2	P:O
1	None	0	DPN	7.3	2.8
	Phosphate 0.02 M	−2		1.3	0
	" 0.02 "	−2	DPN	8.6	1.8
	" 0.02 "	−2	EDTA	1.5	0
	" 0.02 "	−2	DPN + EDTA	6.5	2.4
2	None	0		6.1	3.0
	Phosphate 0.02 M	−2		0	0
	" 0.02 "	−2	DPN	8.0	2.0
	" 0.02 "	−2	Mn^{++}	2.6	0
	" 0.02 "	−2	DPN + Mn^{++}	7.0	2.0
	Arsenate 5×10^{-3} M			0.6	0
	" 5×10^{-3} "		DPN	5.0	2.0
3	None			5.2	2.9
	Arsenite 3×10^{-3} M			0	0
	" 3×10^{-3} "		DPN	6.1	0
4	None	0		7.3	3.1
	Arsenite 3×10^{-4} M	−2		1.3	0
	" 3×10^{-4} "	−2	DPN	7.8	1.9
5	None	0		8.9	3.1
	Zn^{++} 3×10^{-5} M	−2		2.4	0.8
	" 3×10^{-5} "	−2	TPN	6.7	0
	" 3×10^{-5} "	−2	DPN	8.8	1.0

Restoration of Oxidation and Phosphorylation after Preincubation with Various Agents—Many substances and combinations of substances have been tested for ability to restore oxidation and phosphorylation with β-hydroxybutyrate. Occasionally ATP had a slight effect, and in one or

two cases Mn^{++} produced a small but significant effect on oxidation (Table V). Otherwise the results were entirely negative, with one striking exception. DPN in moderately high concentrations (1×10^{-3} M) completely restored the oxygen consumption to the control level and restored phosphorylation 60 per cent or better (occasionally 80 to 90 per cent) after preincubation with phosphate. Restoration of phosphorylation was often less, but still very significant after arsenate, arsenite, sulfate, Ca^{++}, Zn^{++}, saponin, and deoxycholate. It was only after the higher concentrations of arsenite, deoxycholate, and lauryl sulfate that DPN was unable to restore any phosphorylation.

Mn^{++} added with DPN did not improve the restoration of phosphorylation, but in many cases EDTA and DPN together resulted in complete restoration of oxidation and a P:O ratio very near to that of the corresponding controls, especially after preincubation with phosphate.

Recently Lindberg and Ernster (12) reported similar restoration of oxidation by DPN and ATP after preincubation with Ca^{++}, but they found added Mn^{++} essential for restoration of phosphorylation. Because of great differences in the medium and preincubation conditions in the two studies, a number of attempts were made to duplicate these observations. Using our medium, we were never able to obtain any greater effect with DPN and Mn^{++} than with DPN alone. Using their preincubation procedure and medium, with the exception of a less marked hypertonicity, we were able to confirm their results partially, demonstrating an increase in phosphorylation with Mn^{++} in two experiments, but no effect in three experiments. The experimental differences responsible for these variations are unknown, but it is apparent that Mn^{++} may play a fundamental rôle.

TPN can replace DPN to a certain degree in restoration of oxidation. However, there is little or no restoration of phosphorylation with TPN (Table V). The possibility of a phosphorylation coenzyme in the DPN was considered. However, experimental work suggests that the most likely explanation is that TPN is converted to DPN, and that the lag period while this is occurring plus an inhibitory effect of TPN or an impurity in TPN results in inactivation of the phosphorylating mechanism.

DISCUSSION

Preincubation of mitochondria with a number of substances of rather diverse nature seems to produce basically the same result. Morphological changes cause a decrease in turbidity of the suspension similar to that seen when mitochondria swell in water. Although Cleland (13) has ascribed such changes with phosphate to permeability to the divalent ion, this explanation seems unlikely for low concentrations of phosphate added to mitochondria in 0.25 M sucrose.

A striking biochemical change produced by preincubation of mitochondria with various substances is the inactivation of oxidations involving electron transfer via DPN. Simultaneously there is a loss from the mitochondria of material with an absorption maximum at 260 $m\mu$[2] (see also (11, 14)). This undoubtedly includes DPN and split-products of DPN, for the oxidations can be restored completely and phosphorylation restored to a large degree by adding DPN. The material with the E_{260} absorption probably includes other coenzymes, for DPN, AMP, ADP, and ATP accounted for only 30 per cent of it. The loss of AMP, ADP, ATP, Mg^{++}, and K^{+} may also be critical, but this would not be detected in the oxidative phosphorylation test because these substances are part of the test incubation medium. Other biochemical changes such as increases in "ATPase" (1), loss of a small amount of protein,[2] or loss of other nucleotides may be responsible for the incomplete restoration of phosphorylation.

DPN might be lost by diffusion through a more permeable damaged membrane. However, suspension in water or 0.02 M Tris can result in similar or greater swelling without loss of DPN-dependent oxidations and phosphorylations. It seems unlikely that the DPN is free to diffuse out if it does not become limiting with water-swollen mitochondria. The presence of bound DPN in "cyclophorase" and its "discharge" by several agents have been reported (11). However, the experimental support for bound DPN in mitochondria is hardly distinguishable from that for containment of the DPN by a membrane, since nearly every procedure which alters the structure of the mitochondria also results in the "discharge" of DPN from bound sites.

The DPN released is largely split, for the total DPN of the suspension decreases and the released nucleotides contain very small amounts of intact DPN. Whether this splitting occurs in the process of discharge from bound sites, is the result of diffusion to the site of DPNase or nucleotide pyrophosphatase, or is due to activation of DPNase remains to be determined, but the inhibitor nicotinamide does give partial protection.

The question of how the various substances act to produce basically the same effect is a fundamental one. Each might act by damaging the mitochondrial membrane in a different way, but it is less clear how they might all act to discharge DPN from bound sites. Equally puzzling is the observation that the same substances protect against three types of damaging agents. A number of observations raise the question whether there might be a common mechanism involving a trace metal impurity. According to this hypothesis certain metals might displace but not substitute for Mg^{++} or Mn^{++} in the enzyme-metal-coenzyme complex, resulting in the release of Mg^{++} or Mn^{++} and bound DPN. This concept is con-

[2] Unpublished experiments.

sistent with suggestions of Raaflaub (3), Siekevitz and Potter (14), and Lindberg and Ernster (12). ATP and ADP might protect by forming complexes with metals rather than through any specific coenzyme or energy-rich phosphate function.

Metal impurities, however, appear not to be the correct explanation for the observed effects with phosphate. Attempts to remove such impurities by recrystallization from EDTA, treatment with 8-hydroxyquinoline and recrystallization, and passage through the hydrogen form of cation exchange columns to remove all metal ions failed to change the effect of phosphate. Also passage through the hydroxyl form of anion exchange columns with complete removal of phosphate did not yield a solution of inhibitory cations.[3] Tris, which inhibits bisulfite oxidation catalyzed by metals in phosphate, does not protect mitochondria against phosphate. Thus, it appears likely that the phosphate ion itself produces effects very similar to Zn^{++}, etc.

A reasonable explanation for a direct effect of phosphate would be interaction with Mg^{++} or Mn^{++} essential in the enzyme-metal-coenzyme complex. Some surface-active agents might act by a similar mechanism. The similarity between complex formation or removal of Mg^{++} or Mn^{++} by phosphate or other substances and displacement by Zn^{++} or Ca^{++} is obvious. This hypothesis would explain why these substances produce similar effects. Other possible mechanisms cannot be ignored. Phosphate might compete with DPN for binding sites (14), especially in the absence of Mg^{++}. Phosphate might cause a phosphorolysis which splits off DPN or even splits the DPN molecule. The similar actions of arsenate and arsenite are suggestive in this regard. Release of DPN by surface-active agents might be basically similar to the effect of lauryl sulfate in dissociating nucleoprotein (15).

If phosphate and related ions produce their effects directly, the protective effect of EDTA must be due to some basic effect on the mitochondria, rather than to complex formation with metal impurities in the medium. This might involve some complex with the coenzyme-metal-protein, preventing removal or displacement of the metal and dissociation of DPN. ATP might act similarly.

If the primary effect of various agents were displacement of a metal essential in phosphorylation, with release of DPN an almost concomitant secondary effect, phosphorylation might decline slightly more rapidly than oxidation, as it does, and DPN alone might restore only oxidations, as found by Lindberg and Ernster (12). In our experiments Mg^{++} may have sufficed for restoration of phosphorylation, for the Ca^{++} or other agent

[3] The experimental work designed to determine whether the effects were due to phosphate itself or to a metal impurity was carried out by Mr. J. F. Levy.

was always diluted out in the test incubation. In Lindberg and Ernster's experiments the very high concentration of some of the constituents of the medium may have prevented Mg^{++} from being effective, and hence the more powerful Mn^{++} was required.

SUMMARY

1. Preincubation of rat liver mitochondria with phosphate, arsenate, arsenite, pyrophosphate, low concentrations of several metal ions like Zn^{++}, higher concentrations of Ca^{++}, or low concentrations of saponin, deoxycholate, and lauryl sulfate causes a decrease in the turbidity of the suspension and inactivation of DPN-dependent oxidations.

2. These effects are prevented in nearly every case by EDTA, Mn^{++}, Mg^{++}. Some protective effects are also seen with citrate, ATP, ADP, AMP, DPN.

3. DPN, added to a test system already containing ATP and Mg^{++}, completely restores the oxygen consumption and restores phosphorylation 60 to 90 per cent after phosphate, less after some of the other agents.

4. Mn^{++} added with DPN did not raise the P:O ratio above that produced with DPN alone, but EDTA nearly always increased the P:O ratio somewhat.

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Note added in proof. Further studies indicate that the inactivating effect of phosphate is seen only under aerobic conditions.

SERINE AND FORMALDEHYDE AS METABOLIC PRECURSORS FOR THE NICOTINE *N*-METHYL GROUP*

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In tobacco plant metabolism the α-carbon of glycine was incorporated into the *N*-methyl group of nicotine to a greater extent than either formate or the methyl group of methionine when these three compounds were administered under essentially identical conditions (1, 2). As a result of this finding it was concluded that a possible intermediate in the conversion of glycine to methyl groups was a 1-carbon compound at the oxidation state of formaldehyde. It was, therefore, considered of interest to compare the utilization of the α-carbon of glycine for methyl group formation with compounds which might be in the metabolic pathway of glycine to the methyl group. Formaldehyde and serine were chosen as two such compounds. The β-carbon of serine was shown previously to be a precursor of methyl groups in animal metabolism (3, 4), and it was postulated that in the rat a possible route for incorporation of the α-carbon of glycine into choline methyl groups might be through the β-carbon of serine. Likewise formaldehyde was shown to be a precursor of choline methyl groups in the rat (5–7).

The present work demonstrates that both formaldehyde and the β-carbon of serine are rapidly introduced into the methyl group of nicotine in photosynthesizing tobacco plants. In comparison with the α-carbon of glycine, formaldehyde is utilized to a greater extent for methyl group synthesis, whereas the β-carbon of serine is utilized to a slightly lesser extent.

EXPERIMENTAL

Nicotiana rustica L. about 3 months old was used as the experimental plant. The plants were grown in the greenhouse, and the possible methyl group precursors were administered from a nutrient solution as described previously (1).

Uptake of DL-*Serine*—Before administration of the radioactive serine to the plants it was necessary to ascertain whether the amino acid was

* This work was done under contract No. AT(11-1)-161 with the Atomic Energy Commission.

toxic in low concentrations or whether microorganisms on the roots might destroy or change the serine before absorption. Each of six tobacco plants was therefore fed 2 mg. of DL-serine in an Erlenmeyer flask containing about 50 ml. of nutrient medium prepared as described by Byerrum and Wing (8). After 2 days the plants were removed from the flasks, and the solution in each was analyzed for remaining serine with the ninhydrin method of Harding and MacLean (9). This somewhat non-specific procedure was considered adequate, since it was shown that plants growing in the nutrient solution containing no added serine excreted nothing which gave a color with ninhydrin under the conditions employed. The results of the analyses showed that in 2 days 95 per cent of the serine originally present had disappeared from the nutrient medium of each plant. That this disappearance represented absorption of serine by the roots and not destruction by microorganisms was indicated by the finding that, when four flasks with nutrient medium containing 2 mg. of serine were inoculated with about six tobacco root fragments approximately 1 cm. in length, no decrease in the quantity of serine was noted in a 2 day period compared with results in uninoculated controls.

The DL-serine in the concentration used was not toxic to the plants, since normal increases in the length of stems, production of leaves, and growth of roots were observed. These findings are in accord with the results of Ghosh and Burris (10) which indicate that tobacco plants may utilize DL-serine for growth and nitrogen metabolism when fed in a nutrient solution in the presence of ammonium ion.

Uptake of Formaldehyde—It was also of interest to observe whether formaldehyde might be taken up by plants from a nutrient solution and whether it might be toxic when administered in low concentrations. Since formaldehyde is volatile, it was necessary to take some precautions against this loss. Two tobacco plants were placed individually in Erlenmeyer flasks containing 1.7 mg. of formaldehyde in about 50 ml. of the nutrient medium. A plug of cotton was inserted in the neck of the flask around the stem of the plant to minimize loss of formaldehyde through volatilization. The plants were grown for 2 days, after which time the nutrient medium of each plant was analyzed for remaining formaldehyde by a colorimetric procedure which depended on the color formed by the reaction of formaldehyde with phenylhydrazine and potassium ferricyanide (11). After the 2 day period it was indicated that 89 per cent of the original formaldehyde had disappeared from each nutrient solution. It was considered possible that this disappearance represented a reaction between formaldehyde and the proteins of the root surface and not true absorption. That this was not the case was shown by two experiments. The first was carried out by placing about six root fragments 1 cm. in

length in 50 ml. of nutrient medium with 1.7 mg. of formaldehyde. After 2 days there was no detectable decrease in the formaldehyde content of the medium compared with results for controls not containing root fragments. In the controls in which formaldehyde and nutrient solution were incubated for 2 days there was essentially no loss of formaldehyde through volatilization. The second experiment showed that when radioactive formaldehyde was fed in the nutrient medium radioactivity spread rapidly through the plants. It was therefore concluded that formaldehyde in the concentrations used was taken up nearly quantitatively through the roots in a 2 day period.

That these concentrations of formaldehyde had no adverse effect on the plants was indicated by normal growth and appearance.

TABLE I

Incorporation of Formaldehyde and β-Carbon of Serine into N-Methyl Group of Nicotine

Experiment No.	Compound fed	Specific activity of administered compound (*a*)	Specific activity of nicotine dipicrate (*b*)	Dilution $\left(\frac{a}{b}\right)$	Specific activity of methyltriethylammonium iodide
		c.p.m. per mmole	*c.p.m. per mmole*		*c.p.m. per mmole*
1	Serine	6.42×10^6	4.1×10^3	1570	3.5×10^3
2	"	6.28×10^6	2.2×10^3	2860	2.1×10^3
3	Formaldehyde	1.84×10^7	3.9×10^4	470	
4	"	1.84×10^7	5.7×10^4	320	5.6×10^4
5	"	0.77×10^7	0.9×10^4	860	0.8×10^4

Radioactivity of Nicotine from Plants Fed DL-*Serine-3-C*^{14}—DL-Serine-3-C^{14} was fed to two groups of tobacco plants under conditions previously described (1).[1] Each plant received 1.6×10^{-5} mole (1.66 mg.) of serine having a total radioactivity of 1×10^5 c.p.m. when measured with a thin end window Geiger-Müller tube and a Nuclear Instrument and Chemical Corporation scaler. The efficiency of the tube as used was 2.0 per cent.

After a 7 day growing period, nicotine was isolated from the plants as the dipicrate (1). The results of measurement of the nicotine dipicrate for radioactivity are presented in Table I. The radioactivity is expressed as counts per minute per millimole at infinite thinness. It will be noted that an average specific activity of about 3.1×10^3 c.p.m. per mmole was obtained. The dilution of the isotope in the conversion of serine to nicotine (Table I) was assumed to be an expression of the extent of utilization of the β-carbon of serine for synthesis of the *N*-methyl group of nicotine.

[1] The DL-serine-3-C^{14} was obtained from the California Foundation for Biochemical Research, Los Angeles, and the formaldehyde-C^{14} was obtained from the Isotopes Specialties Company, Inc., Glendale, California.

The reason for the difference of nearly 2-fold in the specific activity of the nicotine in Experiments 1 and 2 is not known, but may be the result of seasonal variations in growth and metabolism. The plants in Experiment 1 were grown in the summer, whereas those in Experiment 2 were grown in the fall.

Since the nicotine was radioactive, it was considered of interest to ascertain the fraction of the total radioactivity located in the *N*-methyl group. For such measurements nicotine was recovered from the dipicrate by azeotropic distillation with water from an alkaline medium. The nicotine was then demethylated as described previously (1). The methyl iodide obtained in the demethylation reaction was allowed to react with triethylamine to yield methyltriethylammonium iodide, which was counted. It will be noted (Table I) that after feeding serine-3-C^{14} about 90 per cent of the radioactivity of the nicotine was located in the methyl group.

Radioactivity of Nicotine from Plants Fed Formaldehyde-C^{14}—Three groups of plants were fed formaldehyde-C^{14} for 7 days,[1] after which the nicotine was isolated as the dipicrate. In Experiments 3 and 4 each plant received in 50 ml. of the nutrient solution 1.3×10^{-5} mole (0.39 mg.) of formaldehyde having a radioactivity of 2.39×10^5 c.p.m. In Experiment 5 each plant received the same molar quantity of formaldehyde as in Experiments 3 and 4, but the radioactivity was 1×10^5 c.p.m. It will be noted that the moles of formaldehyde and serine fed per plant were similar.

The radioactivity of the nicotine dipicrate isolated from the three groups of plants is also presented in Table I. It will be seen that dilutions between 320 and 860 were obtained for the isotope in the conversion of formaldehyde to nicotine. DL-Serine-3-C^{14} was diluted from 1570 to 2860 times when administered under essentially the same conditions. If the dilution is considered to be a measure of the extent of utilization, it may be concluded that formaldehyde is used to a greater extent than the β-carbon of serine for nicotine methyl group synthesis.

Again a variation in the radioactivity of the nicotine in Experiments 3, 4, and 5 was noted. This was probably due in part to seasonal variations in metabolism. It is also possible that differences in size of the nicotine pool at the time of feeding the radioactive compounds could affect the radioactivity of the nicotine isolated to the extent shown in Table I, but actually the magnitude of this effect is unknown.

Since the nicotine was radioactive after feeding the formaldehyde, it was of interest again to ascertain the amount of the total radioactivity located in the *N*-methyl group. The nicotine was therefore demethylated and the methyl group recovered as methyltriethylammonium iodide. It will be noted that over 90 per cent of the radioactivity of nicotine from plants fed formaldehyde-C^{14} was located in the *N*-methyl group.

DISCUSSION

The results of the present study demonstrate that both formaldehyde and the β-carbon of serine are precursors of the *N*-methyl group of nicotine in tobacco plant metabolism. Although the importance of formaldehyde and the β-carbon of serine in methyl group formation in plants is reported for the first time, these compounds previously have been demonstrated to be precursors of methyl groups in animals. Du Vigneaud, Verly, and Wilson (5) and Jonsson and Mosher (6) showed that formaldehyde may serve as a precursor for the methyl groups of choline in rats, whereas Weissbach, Elwyn, and Sprinson (3) and Jonsson and Mosher (6) demonstrated the utilization by the rat of the β-carbon of serine for choline methyl group synthesis. Arnstein (4) indicated that in animal metabolism of the two isomers only L-serine yielded a 1-carbon unit for methyl group synthesis. In the present study DL-serine was used, and in consideration of the utilization of the amino acid it was assumed that both isomers yielded methyl groups. The rôle of the D form in plant metabolism is not yet determined and, therefore, further study will be needed to settle this point.

In previous studies (1, 2, 8, 12–14) the methyl groups of choline, glycine betaine, and methionine, the carbon of formate and the α-carbon of glycine were found to be precursors of methyl groups in various plant products. In experiments with nicotine formation in tobacco in which a comparison could be made, it appeared that the α-carbon of glycine was utilized for the synthesis of nicotine methyl groups to a greater extent than were any of the other substances. Nicotine isolated from plants fed glycine-2-C^{14} was about twice as radioactive as nicotine from plants fed methionine methyl-C^{14}. The methyl groups of choline and methionine were transferred to the nicotine *N*-methyl group in approximately equal amounts, whereas formate was introduced at about one-tenth the extent of methionine.

It is also of interest to compare the incorporation of formaldehyde and serine with the other precursors fed formerly. Formaldehyde seemed to enter the methyl group of nicotine in about 3 to 4 times the quantity of the β-carbon of DL-serine when the two were administered under similar conditions. The extent of incorporation of the β-carbon of serine was slightly less than that of the α-carbon of glycine and about the same as for the methyl group of methionine. It is apparent, therefore, that of all the substances fed thus far formaldehyde is utilized to the greatest extent for nicotine methyl group synthesis.

The relative importance in animal metabolism of all the methyl group precursors mentioned above has been ascertained. The methyl groups of betaine and methionine seem to supply most of the methyl groups in vari-

ous animal products by way of transmethylation (15, 16). In the *de novo* synthesis of methyl groups in rats, Arnstein and Neuberger (17) have shown that formate enters the methyl group of methionine to the same or a somewhat greater extent than does the β-carbon of serine. Serine-3-C^{14} and glycine-2-C^{14} fed in equal molar quantities resulted in about 6 times more methyl groups in methionine from serine than from glycine. Stekol *et al.* (16) have also demonstrated that the β-carbon of serine enters the methyl groups of choline in greater amounts than does either formate or the α-carbon of glycine. Formaldehyde and formate, in experiments in which a comparison could be made, appeared to enter methyl groups to a similar extent (5, 7).

Formaldehyde and glycine, therefore, seem to be more important from a quantitative standpoint in methyl group formation in plants than they are in methyl group synthesis in animals.

The present studies shed some light on possible pathways for methyl group formation in plants from the compounds fed. It appears that in the synthesis of the *N*-methyl group of nicotine neither formaldehyde nor the α-carbon of glycine is metabolized to a major extent by way of the β-carbon of serine. It has been postulated previously that in animal metabolism the α-carbon of glycine may enter methyl groups through serine (17, 18).

The hypothesis that formaldehyde, the β-carbon of serine, and the α-carbon of glycine might give rise to an active 1-carbon unit at the oxidation state of formaldehyde, similar to the compound proposed by Berg (19) or Kisliuk and Sakami (20), is attractive. This active 1-carbon compound could then be reduced either to active methyl groups for transmethylation or to "non-labile" methyl groups, such as occur in lignin. In support of the idea that serine may give rise to formaldehyde is the observation in the comparison studies that the β-carbon of serine cannot be converted to methyl groups by way of formate to any great extent. This finding is in line with the discovery of Elwyn, Weissbach, and Sprinson (21) that serine is not oxidized to formate in the formation of methyl groups in the rat.

On the other hand, the possibility exists that the β-carbon of serine may be metabolized to form methyl groups by a different pathway than that of glycine or formaldehyde, as has been suggested by Stekol *et al.* (22) in animal metabolism.

It is interesting to consider the rôle of formaldehyde in plant metabolism. Formaldehyde was postulated by Baeyer (23) to be the reduction product of carbon dioxide in photosynthesis, which then reacted further to yield carbohydrates and other plant products. This concept has been convincingly disproved in recent experiments which indicate that in photo-

synthesis carbon dioxide is introduced into plant metabolism by carboxylation of a 2-carbon intermediate to yield phosphoglyceric acid (24). In addition Ruben *et al.* (25) were not able to demonstrate any radioactive formaldehyde after giving plants radioactive carbon dioxide. However, the fact that formaldehyde is metabolized so readily in tobacco plants suggests that it, or a closely related compound, might be a normal intermediate in plant reactions. If formaldehyde is a normal intermediate, it would seem to function as a precursor of methyl groups or of other compounds derived from the 1-carbon pool. It would further appear that formaldehyde is not derived from the direct reduction of carbon dioxide either in plants or animals, since there is no evidence that carbon dioxide can be reduced to formate or any other 1-carbon compound (5, 26, 27). It would seem reasonable to postulate that a 2-carbon compound, possibly in equilibrium with glycine, might split to give a 1-carbon unit at the oxidation state of formaldehyde. It is entirely possible that if such a reaction occurs free formaldehyde is never formed, at least not in detectable concentrations, but rather that "active formaldehyde" (19, 20) might be the product produced. A 1-carbon intermediate of this type has been suggested for the reaction in which formaldehyde is converted to the β-carbon of serine in animals (7).

SUMMARY

1. Formaldehyde and the β-carbon of serine were incorporated into the *N*-methyl group of nicotine in tobacco plant metabolism.

2. Three to four nicotine methyl groups were formed from formaldehyde for each one formed from the β-carbon of serine when the two precursors were fed under similar conditions.

3. The significance of these results with regard to the mechanism of synthesis of the *N*-methyl group of nicotine is discussed.

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THE SEPARATION OF SPHINGOSINE AND RELATED COMPOUNDS BY REVERSED PHASE PARTITION CHROMATOGRAPHY*

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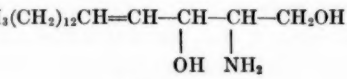
An outstanding limitation to the study of the metabolism of lipides has been the difficulty of assay and isolation of small quantities of the individual lipide components. In addition, the classical methods for the separation of lipides of similar but not identical composition require large quantities of material and are only moderately successful when the structural differences between the compounds are small. Chromatography offers the possibility of separating closely related materials. Recoveries are high, and minor constituents are not apt to be overlooked. The isolation of lipides from small samples of tissue is facilitated, since pure compounds are obtained from a crude extract by a single operation.

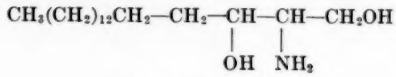
We have developed a procedure for the separation of sphingosine and related compounds by reversed phase partition chromatography. The success of the separation depends on the conversion of the sphingosine-like compounds to an acidic derivative. Excellent separations are achieved. With this procedure it should be possible to discover as yet unknown compounds related to sphingosine.

Three sphingosine-like compounds are known to occur in nature. These are sphingosine, dihydrosphingosine, and phytosphingosine (cerebrin base). Although sphingosine was discovered in 1901 (1), the structure of the three compounds has only recently been definitively established by the elegant work of Carter and his coworkers (2–5):

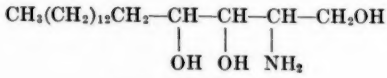


Sphingosine

* This investigation was supported in part by a research grant (No. B-242(C2)) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service, and in part by a grant from the National Science Foundation. A preliminary report of this work was presented before the Forty-sixth annual meeting of the American Society of Biological Chemists at San Francisco, 1955, and at a symposium on neurochemistry and related topics at Atlantic City, 1954.

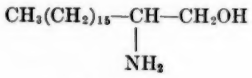


Dihydrosphingosine

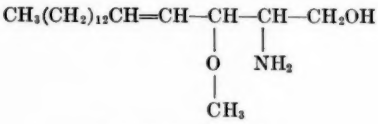


Phytosphingosine

In addition, two other compounds not found in nature have been used in this investigation. Sphingine (3) is a synthetic compound differing from dihydrosphingosine in lacking the hydroxyl group at position 3. *O*-Methylsphingosine (6) is an artifact produced during the hydrolysis of sphingolipides with acid methanol.



Sphingine

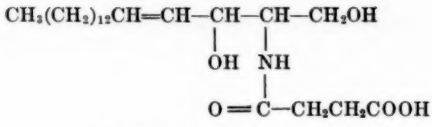


O-Methylsphingosine

Sphingosine occurs as a constituent of a wide variety of animal lipides (7). Dihydrosphingosine has been found in beef spinal cord cerebrosides (8), in liver sphingomyelin (9), and in the lipides of larval *Cysticercus fasciolaris* (10). Phytosphingosine has been found only in plant sources: in corn and soy bean phosphatides (5), in molds (11, 12), in yeast (13), and in mushrooms (14). Possibly other as yet unknown sphingosine-like compounds occur in tissues.

In attempting to separate the sphingosine-like compounds, we were encouraged to try partition chromatography by the success which many workers have had in separating fatty acids by solvent partition, both by counter-current distribution (15, 16) and by chromatography (17–21).

The free bases, however, did not appear to be particularly well suited to this type of separation. Therefore, prior to chromatography, the sphingosine and related compounds are converted to their *N*-succinyl derivatives by reaction with succinic anhydride, thus introducing a carboxylic acid group into the molecule.



N-Succinylsphingosine

The reaction was found to be rapid and quantitative. Under the conditions used, the hydroxyl groups of sphingosine do not react. Titration of the carboxyl group of the *N*-succinyl compounds provides a rapid assay for sphingosine. The results obtained by this assay are presented in Table I.

TABLE I

Conversion of Dihydrosphingosine to N-Succinyldihydrosphingosine

Dihydrosphingosine added	Succinyldihydrosphingosine formed	Recovery
μmoles	*μmoles*	*per cent*
0	0	
0.88	0.60	68
1.8	1.4	78
3.5	3.4	97
5.29	5.05	95
7.05	6.70	95
8.82	8.20	93
17.6	17.9	102
26.4	27.0	102
35.3	36.1	102
44.1	44.4	101
88.2	88.4	100
132	130	99

The properties of the *N*-succinyl compounds determined the conditions that could be used for their chromatographic separation. Their limited solubility restricted the choice of solvents to those containing both chloroform and alcohol. In these solvent systems the *N*-succinyl compounds were found to have partition coefficients in favor of the less polar, chloroform-rich phase. This demanded the use of reversed phase partition chromatography, in which the less polar phase is stationary.

Reversed phase chromatograms were pioneered by Boldingh (18), using powdered rubber as a supporting phase, and by Howard and Martin (19), who introduced a supporting phase of diatomaceous earth rendered nonwettable to polar solvents by treatment with dichlorodimethylsilane. However, Howard and Martin had found that columns prepared with the chloroform-aqueous methanol solvents which we were forced to use were particularly unstable and tended to lose the stationary phase during use.

This difficulty was resolved by Bergström and Sjövall (22), who found that the addition of 10 per cent of heptane would cause the chloroform phase to adhere to the supporting phase. Presumably the hydrocarbon acts by increasing the interfacial tension of the two phases (19). We have found hydrocarbons of higher molecular weight to be more effective, and use isooctane or Skellysolve S, a decane fraction.

The system adopted has as its stationary phase chloroform-hydrocarbon, which is supported on siliconized diatomaceous earth. The moving phase is aqueous methanol. In this system sphingosine-like compounds differing in the number of their hydroxyl groups are widely separated (Figs. 1 and

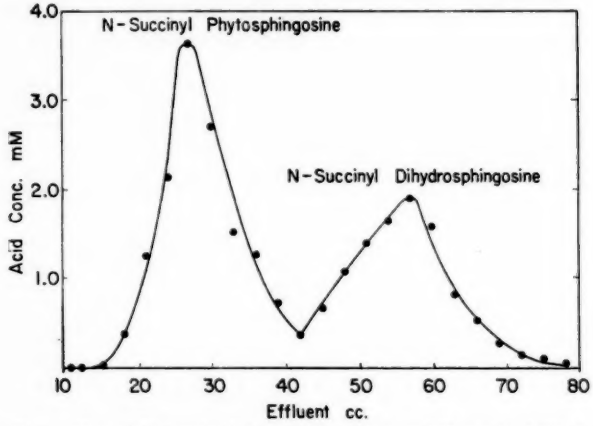


FIG. 1. Separation of *N*-succinylphytosphingosine and *N*-succinyldihydrosphingosine on a column 1.8 × 11.1 cm. Solvent system: chloroform-Skellysolve S-methanol-water, 8:1.5:20:12 (volume by volume); 0.05 mmole of each compound.

2). The most polar succinylphytosphingosine (three hydroxyl groups) emerges first, followed by succinyldihydrosphingosine (two hydroxyl groups). Succinylsphingine (one hydroxyl group) emerges last. Compounds differing in the methylation of a hydroxyl group are separated (Fig. 3); succinyldihydrosphingosine emerges ahead of succinyl-*O*-methylsphingosine. A partial separation of a substituted from an unsubstituted compound is shown in Fig. 4. This last separation, however, serves more to define the limits of the method than as a practical procedure.

EXPERIMENTAL

Materials—Solvents were used without special purification. Isooctane (2,2,4-trimethylpentane) is manufactured by the Phillips Petroleum Company, Bartlesville, Oklahoma. Skellysolve S (boiling range, 150–202°) is

the product of the Skelly Oil Company, Kansas City, Missouri. It is stated by the manufacturer to contain some nonanes and undecanes and a

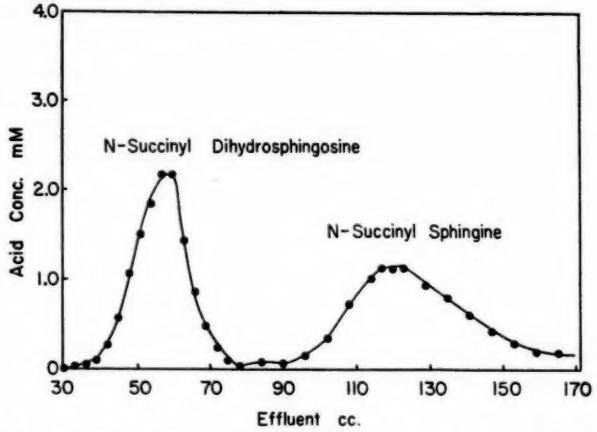


FIG. 2. Separation of *N*-succinyldihydrosphingosine and *N*-succinylsphingine on a column 1.8 × 10.4 cm. Solvent the same as in Fig. 1; 0.05 mmole of each compound.

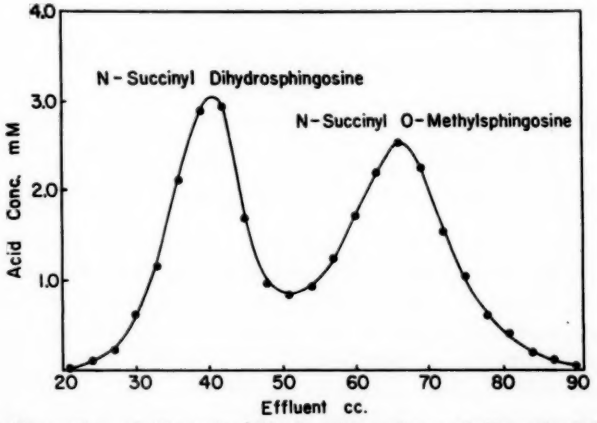


FIG. 3. Separation of *N*-succinyldihydrosphingosine and *N*-succinyl-*O*-methylsphingosine with a fast running solvent. Column dimensions, 1.8 × 10.9 cm. Solvent system: chloroform-Skellysolve S-methanol-water, 8:1.75:20:11 (volume by volume); 0.05 mmole of each compound.

considerable quantity of normal and isodecanes. Dichlorodimethylsilane (purified grade) was purchased from the Dow Corning Corporation, Mid-

land, Michigan. Hyflo Super-Cel, a purified diatomaceous earth, is the product of Johns-Manville. Commercial succinic anhydride was recrystallized from acetic anhydride before use.

Sphingosine was obtained by hydrolysis of triacetylsphingosine (m.p. 99–101°, $[\alpha]_D^{25}$ −11.6°; Carter *et al.* (8) record m.p. 101–102°, $[\alpha]_D^{25}$ −11.7°), prepared from beef brain cerebrosides (23) by the method of Carter *et al.* (8). Dihydrosphingosine was obtained by hydrolysis of tribenzoyldihydrosphingosine (m.p. 144–145°; Carter *et al.* (8) record 144–145°) prepared from beef spinal cord cerebrosides (23). Phytosphingosine was obtained

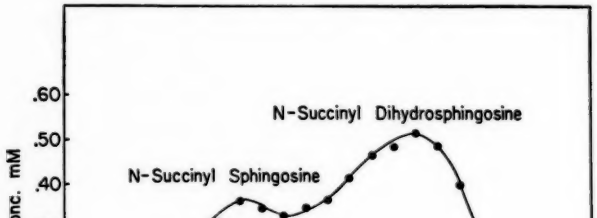


FIG. 4. Partial separation of *N*-succinylsphingosine and *N*-succinyldihydrosphingosine on a column 1.8 × 8.4 cm. Solvent system: chloroform-1,2-dichloroethane-isooctane-methanol-water, 4:4:1:20:16 (volume by volume). 0.015 mmole of *N*-succinylsphingosine; 0.02 mmole of *N*-succinyldihydrosphingosine.

by hydrolysis of its acetone compound (m.p. 110–112.5°; Carter *et al.* (5) record 105–107°) prepared from corn phosphatides (5). We wish to thank Dr. H. E. Carter for his gifts of synthetic *dl*-sphingine sulfate and of *O*-methylsphingosine hydrochloride, isomer II. The preparation of the *N*-succinyl derivatives is described below.

Reaction with Succinic Anhydride—A sample containing from 1 to 100 μmoles of sphingosine is dissolved in 5 cc. of a solution of 1 per cent of sodium acetate dihydrate in methanol, and transferred to a 30 cc. stoppered, conical bottomed centrifuge tube. The solution should be alkaline to litmus. Two 0.5 cc. portions of a saturated solution of succinic anhydride in acetone (0.16 gm. per cc.; 1.6 M) are added 5 minutes apart. After 10 minutes, 1 cc. of methylene bromide (CH_2Br_2) and 5 drops of glacial acetic acid are added; the solution is mixed, and about 25 cc. of water are added.

The precipitated *N*-succinyl derivatives are centrifuged, the water layer removed by aspiration, and the combined precipitate and methylene bromide washed twice with 25 cc. portions of water. The various compounds studied behave somewhat differently in the precipitation. *N*-Succinylsphingosine and *N*-succinyldihydrosphingosine form dense curds which settle out immediately. *N*-Succinylphytosphingosine forms a colloidal suspension which must be stored in the cold for several hours to allow aggregation of the particles before it is centrifuged. *N*-Succinylsphingine forms a colloidal suspension which becomes partly crystalline after several hours in the cold. *N*-Succinyl-*O*-methylsphingosine is largely soluble in the methylene bromide; *N*-succinyldihydrosphingosine is completely insoluble in alcohol-free methylene bromide. The precipitates are dried in a desiccator and are ready for chromatography.

Titration of the carboxyl group of the *N*-succinyl derivatives provides a measure of the total sphingosine-like compounds present in the sample. The washed precipitated *N*-succinyl derivatives are brought into solution by the addition of 4 cc. of 95 per cent ethanol to the combined precipitate and methylene bromide, and are titrated as described later. The data in Table I establish the quantitative nature of the reaction and demonstrate its suitability as an assay for sphingosine.

N-Succinyldihydrosphingosine has not been obtained crystalline, but tends to form stiff gels from all solvents tried. The amorphous material melts at 118–121°.

$C_{22}H_{43}O_5N$.	Calculated.	C 65.79, H 10.79, N 3.49
(401.57)	Found.	" 65.70, " 10.68, " 3.56

Neutral equivalent. Calculated, 401.57; found, 401

The analyses prove the purified products to be a monosuccinyl derivative. The data of Table I show the reaction of 1 mole of succinic anhydride per mole of dihydrosphingosine, thus indicating that di- and trisuccinyl compounds are not formed. The succinyl group is assumed to be located on the nitrogen atom. This assumption is supported by the ready formation of *N*-succinyloctadecylamine (m.p. 123–125°; neutral equivalent, calculated, 369, found, 369), and by the failure of octadecanol-1 to react under these conditions.

Preparation of Columns—The chromatographic tubes used were of the Zechmeister-Cholnoky type with ground joints and coarse sintered glass plates, and had an inner diameter of 1.8 cm.[1]

Hyflo Super-Cel is washed twice by decantation with 3 times its volume

[1] Purchased from the Scientific Glass Apparatus Company, Bloomfield, New Jersey.

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of 0.1 N HCl. Fine particles are removed by repeatedly suspending the Super-Cel in 3 times its volume of tap water, allowing it to settle for 1 to 2 hours, and decanting the supernatant fluid. After five washings, the supernatant fluid is relatively free of fine particles. The Super-Cel is finally washed twice with distilled water, collected with suction on a Büchner funnel, and dried in an oven at 110°. The oven-dried material is siliconized by contact with dichlorodimethylsilane vapors (19). The Super-Cel is stirred while a stream of dry nitrogen, previously drawn through liquid dichlorodimethylsilane, is passed through it. Reaction is complete in 20 minutes. The siliconized Super-Cel is washed once with methanol and repeatedly with 95 per cent ethanol until the washings are free of HCl. The material is dried in an oven at 110° and stored as a dry powder.

For the preparation of a column, 15 gm. of siliconized Hyflo Super-Cel are mixed with 13 cc. of the chloroform-rich solvent in a glass mortar. The resulting dry powder is slurried immediately with about 50 cc. of the methanolic solvent. It is convenient to transfer the slurry to the column in three separate portions, each portion being partially packed before the next is added. Each portion of the slurry is diluted after transfer to the column by nearly filling the column with the methanolic solvent. A closely fitting perforated stainless steel plunger (19) is used to pack the column. The slurry is homogenized and freed from air bubbles by short rapid strokes of the plunger and allowed to settle under air pressure of about 50 mm. of mercury until the solvent has moved 2 cm. The slurry is then tamped by long slow strokes of the plunger. Packing the column is completed by alternate settling under pressure and tamping. The air pressure (about 50 mm. of mercury) is adjusted so that the rate of flow of the solvent through the column is 0.5 cc. per minute, and the column is ready for use.

Preparation of Solvent—The solvents are prepared by mixing the four components. Two phases separate. Considerable heat is evolved and the mixture must be allowed to cool and the two phases equilibrated at room temperature. The lower, chloroform-rich phase serves as the stationary phase on the column. The upper, methanolic phase serves as the moving phase.

The solvent mixtures are described below. Isooctane may be substituted for Skellysolve S in any of the solvent mixtures without change in the resolution of the columns; however, leaching of the stationary phase from the top few cm. of the column will occur.

Operation of Columns—The columns are operated at room temperature. However, the mutual solubility of the two solvent phases is strongly affected by small changes in temperature and it is advisable to minimize fluctuations in room temperature. Columns may be used repeatedly, but must be protected from changes in room temperature if they are kept overnight.

The positions of the chromatographic peaks, although reproducible, are affected by the ambient temperature.

The compounds to be separated are placed on the column in solution in the methanolic phase containing not more than 10 mg. of solids per cc., and are washed into the column with two 1 cc. portions of the methanolic phase. A total of 60 mg. of material (0.15 mmole) may be handled on a column 1.8 cm. in diameter.

The effluent is collected in 3.0 cc. fractions. Each fraction is titrated with 0.05 or 0.1 N NaOH in methanol delivered from a micro burette[2] to the end-point of phenol red. Stirring is accomplished by a stream of CO_2-free air. Titrations are reproducible to 0.001 cc. The blank (0.5 μmole per sample) is accounted for by the acid present in the methanol used to make the chromatographic solvent.

Separation of Mixtures—The most satisfactory solvent mixture, which gives the widest separation of the peaks, consistent with large capacity is chloroform-Skellysolve S-methanol-water 8:1.5:20:12 (volume by volume). The separations obtained with this solvent of three compounds differing in the number of their hydroxyl groups are shown in Figs. 1 and 2. In this system the succinyl-*O*-methylsphingosine peak is centered at 94 cc. of effluent.

For preparative and certain analytical purposes, a column on which the compounds move more rapidly offers an advantage. The solvent giving the most rapid movement of the compounds consistent with separation of the peaks is chloroform-Skellysolve S-methanol-water 8:1.75:20:11. The separation of succinyldihydrosphingosine and succinyl-*O*-methylsphingosine by this solvent is shown in Fig. 3.

Succinylsphingosine and succinyldihydrosphingosine are not separated with the above solvents. They may be partly separated with solvent systems of the compositions chloroform-1,2-dichloroethane-isooctane-methanol-water, 4:4:1:20:16 (volume by volume) or 5:3:1:20:16. This separation is shown in Fig. 4. With these solvent systems it is necessary to pretreat the column by placing 60 mg. of oxalic acid in a few cc. of the methanolic phase on the column, and wash it through with 70 cc. of methanolic solvent. Part of the oxalic acid adheres to the column and evidently blocks sites which otherwise would bind the succinyl derivatives and distort the peaks. The usefulness of these latter columns is limited by low capacity and the poor solubility of the succinyl derivatives in the methanolic phase.

Characterization of Effluent Compounds—The *N*-succinyl compounds are recovered from the effluent fractions by concentration to a small volume

[2] The Gilmont ultramicro pipette-burette, 1 cc. in size, glass part bent 90° close to the reservoir, sold by the Emil Greiner Company, New York, has proved satisfactory.

and precipitation as already described. They may be recognized by the position of their peaks and by the characteristic appearance of their precipitates. No special attempts have been made to characterize the recovered *N*-succinyl compounds. However, samples have been rechromatographed repeatedly without change in the position of their peaks. Recoveries range from 70 to 100 per cent but are usually about 90 per cent.

DISCUSSION

The use of methylene bromide in the precipitation of the *N*-succinyl compounds requires comment. On the addition of water to their alcohol solutions, these compounds separate as coherent gels which may float or sink. They are not easily collected by filtration. Added methylene bromide is occluded by the gel, which becomes very dense and is easily collected and washed by centrifugation. Chloroform cannot satisfactorily be substituted for methylene bromide. With chloroform the water phase becomes hazy, and considerable losses are experienced.

Two modifications in the preparation of the siliconized diatomaceous earth have been found necessary. Fine particles present in the Hyflo Super-Cel were found to bleed from the columns, blocking the sintered glass plate and contaminating the effluent. These are removed by washing (21). The *N*-succinyl compounds were found to be irreversibly adsorbed by untreated siliconized Hyflo Super-Cel. This adsorption can be eliminated by treating the columns with oxalic acid or, better, by washing the Super-Cel with hydrochloric acid prior to siliconizing (24).

Small changes in the water content of the solvent make large differences in the chromatograms. Increasing the water content slows the movements of the bands, causes them to be more widely separated, and makes the bands more diffuse. The smallest water concentration that will give separation of the compounds on the column is water-methanol in the ratio, 11:20 (volume by volume). When the ratio of water to methanol exceeds 13:20 (volume by volume), the bands become diffuse. Optimal separations are obtained with a ratio of water to methanol of 12:20 (volume by volume).

The chromatographic separation of compounds differing in the number of their polar groups has been described above. It would be of interest to know whether compounds differing in the length of the aliphatic chain would also be separated. No such synthetic compounds were available for testing, but a compound, tentatively identified as the 16-carbon atom analogue of dihydrosphingosine, has been chromatographically separated from beef brain dihydrosphingosine. The *N*-succinyl derivative of this material emerges from the column with a peak centered at 36 cc., immediately preceding the dihydrosphingosine peak.

Higher fatty acids move very slowly on these columns and are not eluted by any reasonable volume of solvent.

It is interesting that columns similar to those described here have given excellent separations of two other classes of acidic lipides, the glycerol phosphatides (25) and the bile acids (22, 24). In addition, a similar solvent system has been used for the separation of brain lipides by counter-current distribution (26).

Using this method, we are studying the synthesis of sphingosine *in vitro* and are examining normal and diseased tissues for the presence of higher fatty bases other than sphingosine and dihydrosphingosine.

SUMMARY

Sphingosine and related compounds have been separated by reversed phase partition chromatography on siliconized diatomaceous earth. Prior to chromatography these compounds are converted to their *N*-succinyl derivatives by reaction with succinic anhydride. Compounds differing in the number of their hydroxyl groups, in methylation of one hydroxyl group, and in degree of unsaturation have been separated.

A new assay for sphingosine is proposed. Sphingosine and related compounds are converted to the *N*-succinyl derivative, and the carboxylic acid group thus introduced is titrated with alkali. Quantities of from 3 to 100 μmoles are determined with an accuracy of 5 per cent.

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A METHOD OF HISTIDINE DETERMINATION BASED UPON A MODIFIED KNOOP REACTION. II

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Extended use of the new method of histidine determination first described in 1952 (1) has brought to light certain factors, not at first recognized, which affect significantly the accuracy and reproducibility of its results.

The original description of the method emphasized the necessity of controlling accurately the temperature at which the first step, bromination of the histidine, is carried out. Not then realized was the equal, if not greater, importance of temperature control during the final step: the development of color by the addition of sodium acetate. The effect of temperature variations at this stage is exemplified by experiments in which 0.08 mg. lots of histidine, brominated at the prescribed temperature of 25°, were treated with the acetate mixture in one series at 25° and in another at 19°. The 25° series gave an average photometric density of 0.2070 (s.d. = 0.00205), the other an average of 0.2189 (s.d. = 0.00302). A 6° reduction of temperature increased color production, therefore, by almost 6 per cent. In itself such an increase would be an evident advantage. A counterbalancing disadvantage is that color development is much slower at 19° than at 25° (requiring 25 to 30 minutes as against 14 to 15). In any case the adoption of different temperatures for the two stages of the method would be practically inconvenient. On the whole, therefore, it seems best to carry out the entire procedure in one thermostat at 25°. The essential is that at both stages the temperature be rigidly controlled.

Due attention to this requirement did not suffice to prevent the frequent appearance of anomalous results. These were ultimately recognized to be related to the age of the bromine solution. A month-old solution almost always, and particularly with the smaller quantities of histidine, gave more color than one freshly prepared. This, it seemed possible, might be due somehow to the gradual development, within the aging solution, of small quantities of hydrogen bromide. To test the validity of this conjecture 5 ml. lots of a solution containing, in different experiments, 0.04, 0.08, 0.12, 0.16, 0.24, or 0.32 mg. of histidine were treated *seriatim* with increasing small volumes (not exceeding 0.21 ml.) of KBr solutions of varying strength (up to 1 per cent). Each mixture, together with a control containing no

bromide, was then subjected to the prescribed procedure for histidine determination. The results, expressed as percentage changes of photometric density due to bromide, are recorded graphically in Fig. 1. Those with 0.08 mg. of histidine appear on Curve A, those with 0.12, 0.16, and 0.20 mg. on Curves B, C, and D, respectively, while those with 0.24 and 0.32

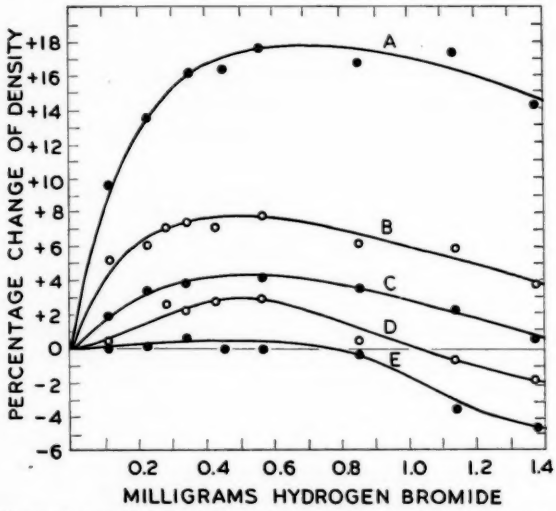

FIG. 1. Effect of bromide upon the photometric density of the color obtained from 5 ml. of solution containing different quantities of histidine. The bromide additions tested are expressed in terms of HBr, and their effects as percentages of the density obtained in the absence of bromide. Curve A is for 0.08 mg. of histidine, Curve B for 0.12, Curve C for 0.16, Curve D for 0.20 mg. Curve E combines the substantially identical results obtained with 0.24 and 0.32 mg. Experiments with 0.04 mg., not represented in the figure, gave a maximal increase, with from 0.34 to 0.86 mg. of HBr, of 29 to 31 per cent. The actual initial (no bromide) densities were, for 0.04 mg. of histidine, 0.0741; for 0.08 mg., 0.1935; for 0.12 mg., 0.326; for 0.16 mg., 0.452; for 0.20 mg., 0.584; for 0.24 mg., 0.701; and for 0.32 mg., 0.925.

mg., being indistinguishable from one another, are combined in Curve E. The curves demonstrate that in the presence of appropriate amounts of bromide (existing, of course, in the acid bromination mixture as HBr) the color is indeed enhanced. The degree of enhancement depends, it is seen, not only on the quantity of bromide, but also on the quantity of histidine present. The smaller the latter, the greater is the effect. Thus, while with 0.24 mg. or more of histidine (Curve E) the increase of color is insignificant, with 0.08 mg. (Curve A) it rises to over 17 per cent, and with 0.04 mg. (not represented in Fig. 1) to about 30 per cent. At all levels of

histidine, the quantity of HBr sufficing to give a maximal effect is substantially the same, about 0.5 mg. Greater quantities lead to a progressive fall of color intensity. The larger the quantity of histidine, the sooner does this fall bring the curve down to, or even below, the level of the bromide-free control. The fall is, however, so gradual that even with 0.75 mg. of HBr the color is still in all cases within 1 per cent of its maximum.

An explanation of these rather peculiar relations is still to be found, but the practical conclusion to be drawn is that to obtain maximal color intensity at all histidine levels bromination should be carried out in the presence of about 0.5 mg. of HBr. KBr sufficient to yield this may be incorporated with the bromine in one solution. The bromide ion concentration

TABLE I

Data for Revised Calibration Curve

Histidine	No. of determinations	Photometric density	
		Mean	S.d.
mg.			
0.04	13	0.0956	0.00143
0.08	8	0.2270	0.00101
0.12	7	0.352	0.00091
0.16	16	0.471	0.00297
0.20	5	0.589	0.00544
0.24	20	0.704	0.00501
0.28	5	0.823	0.00400
0.32	28	0.923	0.00512

of such a solution may be allowed to increase spontaneously by as much as 50 per cent without appreciable effect upon the performance of the mixture.

The bromide, it should be stated, is effective only during bromination. Added later, it has no influence upon the result.

In view of the foregoing and certain subsidiary observations, the original description of the method (1) is to be modified as follows:

(*a*) The *bromine reagent* is to be prepared by dissolving 0.5 ml. of bromine in a mixture of 50 ml. of tertiary butyl alcohol with 50 ml. of a 0.6 per cent aqueous solution of potassium bromide. If this mixture is kept in the dark at 4–5°, it will be serviceable for about a month.

(*b*) For bromination "in a dim light" is to be substituted bromination in a dark room under non-actinic illumination. Daylight is not to be admitted until after treatment with the arsenite reagent.

(*c*) All operations up to the full development of the color (completed

14 minutes after addition of the acetate mixture) are to be carried out at 25° ± 0.2°.

(*d*) The final dilution of the colored mixture to 10 ml. is to be made with water of about 10°, so as to delay, as far as possible, the gradual fading of the color.

The adoption of these modifications gave, for a revised calibration curve, the data shown in Table I.

As might have been expected from Fig. 1, the chief difference from the original calibration curve lies in the greater densities obtained with histidine quantities below 0.2 mg. For quantities of from 0.12 to 0.28 mg. the present data lie on a straight line taking origin from zero, while both above and below that range the line curves downward. The equation for the straight portion (calculated by least squares from 53 individual data) is

$$\text{Mg. histidine} = \text{photometric density} \times 0.34$$

This equation may be used for the interpretation of all found densities between 0.824 and, say, 0.2924 (galvanometer readings 15 to 51). Lower densities down to, say, 0.0915 (galvanometer reading 81) may be referred to a graph, or with equal accuracy, evaluated by the equation

$$\text{Mg. histidine} = \text{photometric density} \times 0.305 + 0.0108$$

derivable from the data for 0.04 and 0.08 mg. of histidine. Densities below 0.0915 are hardly susceptible of exact evaluation.

It is clear that, while the method is good for histidine quantities as low as 0.04 and as high as 0.32 mg., the most reliable results will be obtained when the amount measured lies between 0.12 and 0.28 mg. As far as possible, then, the solution to be analyzed should be so treated, by dilution or concentration, as to bring its histidine content (per 5 ml.) within the latter limits.

SUMMARY

The results of the colorimetric method of histidine determination previously described are shown to be affected by (1) the concentration of bromide ions during bromination and (2) the temperature at which the color is developed. The technique of the method is so modified as to take these factors duly into account.

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EFFECT OF METAL IONS AND ADENOSINE TRIPHOSPHATE ON AN OXALACETATE-INHIBITED SUCCINOXIDASE ACTIVITY*

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In a previous study (1) it was noted that the inhibition of succinate oxidation produced by diphosphopyridine nucleotide (DPN) was relieved by the addition of Mg^{++} to the reaction mixture. This relief occurred despite the fact that the metal ion also caused a simultaneous increase in keto acid production. A few preliminary studies indicated that oxalacetate (OAA) was formed in sufficient quantities to produce profound inhibition; yet it was unable to inhibit succinoxidase activity in the presence of Mg^{++}. Pardee and Potter (2), using a rat kidney homogenate fortified with Mg^{++}, noted that in the presence of adenosine triphosphate (ATP) relatively large amounts of OAA failed to inhibit succinoxidase activity. They offered no explanation for this unusual effect but reported that the delayed addition of Ca^{++} to the reaction mixture almost immediately caused the OAA "to resume the inhibitory form."

This report deals with some factors affecting the ability of OAA to inhibit succinate oxidation. It was found that both Mg^{++} and ATP relieve the inhibition, the combination being very effective. It is also noted that an enzyme which is easily inactivated by preincubation is required for this phenomenon. Furthermore, the effect is not primarily caused by the decarboxylation or utilization of OAA. Finally, it is shown that ethylenediaminetetraacetic acid (EDTA) enhances the action of ATP and Mg^{++}.

Methods

Kidneys removed from decapitated rats were rapidly weighed, homogenized in 0.9 per cent NaCl, and made up to a 5 per cent suspension (wet weight). The elapsed time from killing to placing the vessels in the bath was approximately 5 minutes and care was taken to keep all the reactants ice-cold during that interval. Manometric procedures previously described (1) were used to determine the rate of succinate oxidation. Except when otherwise indicated, the ice-cold enzyme was added directly to the main compartment containing all the substrates. When respiration was not measured, experiments were carried out in small beakers in a Dubnoff

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apparatus. Changes in the concentration of the various keto acids were determined (3) on trichloroacetic acid filtrates of samples taken at stated intervals. The method of El Hawary and Thompson (4), with minor modifications, was used for the paper chromatographic separation and estimation of OAA and pyruvate as their 2,4-dinitrophenylhydrazones. After evaporating the ethyl acetate extracts and taking up the dry residue in NaOH and phosphate buffer, as described (4), the final volume was noted and 0.05 ml. to 0.1 ml. of the solution (depending upon the expected concentration of keto acids) was streaked out over a line 2.5 cm. long on Whatman No. 3 filter paper which was then hung in a solvent composed of *n*-butanol, 70 parts; ethanol, 10 parts; and 0.5 N NH_4OH, 20 parts. The

TABLE I

Effect of Various OAA Concentrations on Rate of Succinate Oxidation

Oxygen data given as microliters of oxygen consumed per mg. of N per hour.

Final concentration of OAA added, $\times 10^{-3}$ M	Oxygen uptake (1st 10 min.)	Per cent inhibition
0.0	755	
0.18	370	51
0.35	140	81
0.70	95	88
1.4	55	93

The Warburg flasks contained, in a final volume of 2.0 ml., 0.5 ml. of a 5 per cent kidney homogenate, cytochrome *c* (4×10^{-5} M), Tris buffer at pH 7.4 (0.02 M), succinate (0.05 M), and OAA in the concentrations indicated. The readings were started after 5 minutes for temperature (38°) equilibration. Gas phase, air.

solvent was allowed to ascend for 15 hours. The identification of the separated OAA and pyruvate 2,4-dinitrophenylhydrazone spots was made by using as markers 2,4-dinitrophenylhydrazones freshly prepared by standard procedures from the pure keto acids and further purified by recrystallization from ice-cold ethyl acetate by the addition of petroleum ether. The different spots were outlined under ultraviolet light, cut out, minced with scissors, and suspended in 5.0 ml. of 10 per cent Na_2CO_3 for 1 hour and then filtered. The concentration of the keto acids in the filtrates of the spots corresponding to the hydrazones of OAA and pyruvate, respectively, was estimated (3), with curves prepared from known amounts of the corresponding keto acids as standards.

OAA was prepared as previously described (1). In a reaction mixture containing succinate (final concentration 0.05 M) the addition of this keto acid to give a final concentration of 0.0007 to 0.0014 M inhibited the initial rate of oxidation of succinate by approximately 90 per cent (Table I). The data given in Table I will be used as a reference for some experiments de-

scribed below dealing with the expected inhibition when various concentrations of OAA are present in the reaction mixture.

The results reported were obtained in Warburg flasks containing 0.5 ml. of whole homogenate (0.7 to 0.9 mg. of nitrogen) in a reaction mixture with a final volume of 2.0 ml. The data presented are typical of a large number of experiments. The values for keto acid changes, when measured by sampling from beakers in a Dubnoff apparatus, agreed closely with

TABLE II

Effect of Various Additions on Oxygen Consumption of Kidney Homogenate

Data expressed as microliters of oxygen consumed per mg. of N per hour.

Line No.	Additions	10 min.	20 min.	30 min.	40 min.	Total keto acids found
						γ per ml.
1	None	5	0	10	0	
2	Succinate	850	840	705	600	1.8
3	" + ATP + Mg^{++}	750	645	455	340	15.0
4	" + OAA	75	80	75	65	125.0
5	" + " + ATP + Mg^{++}	700	615	390	235	115.0
6	" + " + " + " (Ca^{++} tipped in after 10 min. reading)	720	95	60	125	
7	OAA	65	55	30	30	
8	ATP + Mg^{++}	180	85	45	40	
9	OAA + ATP + Mg^{++}	345	255	135	65	
10	Unaccounted, Lines 5 − (8 − 7)	420	415	285	200	
11	" " 5 − (8 + 4)	455	475	315	165	

Additions as indicated above to give final concentrations as follows: ATP (0.002 M), $MgCl_2$ (0.003 M), OAA (0.0014 M), $CaCl_2$ (0.003 M). All vessels contained cytochrome *c*, Tris buffer, and enzyme as described in Table I. Total keto acids determined, where indicated, after stopping the reaction with an equal volume of 10 per cent trichloroacetic acid immediately after last readings. Other conditions as in Table I.

those found in Warburg flasks. In the latter experiments respiration was simultaneously measured and, at stated intervals, a pair of vessels was removed from the bath for keto acid determinations. A few experiments with mitochondria are reported. They were prepared as previously described (5) with the slight modifications given below. Final concentrations and other pertinent details are given in Tables I to VI.

Results

Effect of Various Additions on Oxygen Consumption—The results of typical experiments presented in Table II confirm the findings of Pardee and Potter (2). These data clearly show that ATP and Mg^{++} relieve the inhi-

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bition of succinate oxidation produced by OAA (Line 5). It can also be seen that the delayed addition of Ca^{++} almost immediately restores the ability of OAA to inhibit succinate oxidation (Line 6). In addition to these confirmatory observations, it will also be noted that the action of ATP and Mg^{++} is neither accounted for by changes produced on endogenous O_2 uptake (Lines 10 and 11) nor by the possibility that ATP and Mg^{++} stimulate succinate oxidation *per se*. To the contrary, when added in the absence of OAA, the combination inhibits succinate oxidation (Line 3).

Individual Effects of ATP and Mg^{++}—The results of another series of experiments (Table III) show that, if used alone, either ATP or Mg^{++} can

TABLE III

Effect of Various Additions on Relieving Inhibition Produced by OAA on Succinate Oxidation

Data expressed as microliters of oxygen consumed per mg. of N per hour.

Line No.	Additions	10 min.	20 min.	30 min.	40 min.
1	Succinate	800	760	640	550
2	" + OAA	65	80	45	55
3	" + " + ATP	320	240	140	100
4	" + " + Mg^{++}	280	150	110	100
5	" + " + " + ATP	760	630	400	210
6	" + " + " + " + EDTA	850	650	600	420
7	Succinate + OAA + Mg^{++} + ATP + EDTA (Ca^{++} tipped in after 10 min. reading)	820	105	95	150

Conditions as given in Tables I and II. EDTA used where indicated to give a final concentration of 0.001 M.

relieve the inhibition (Lines 3 and 4). However, considerable variation occurs with different batches of ATP. In most of the experiments reported here it was observed that the response obtained with the combination of ATP and Mg^{++} was slightly higher than the sum of their individual effects (Line 5). The optimal concentration for ATP when used alone was between 0.002 and 0.004 M; that for Mg^{++} was 0.003 to 0.006 M.

Effect of EDTA—The effect of ATP and Mg^{++} in relieving the inhibition is not well maintained and the rate falls off rapidly. This decay is prevented by adding EDTA (Table III, Line 6). Even under such conditions, the delayed addition of an excess of Ca^{++} immediately restores the OAA inhibition (Line 7). It is interesting to note that the chelating agent has little effect, by itself, on an OAA-inhibited system (Table IV, Line 3), but it enhances either the effect of Mg^{++} alone (Lines 4 and 5) or when the

metal ion is used in combination with ATP (Line 8). However, it markedly lowers the effectiveness of ATP when that coenzyme is used alone (Lines 6 and 7). This may indicate that ATP requires, in part, a metal ion that can be removed by EDTA.

Effect of Various Metal Ions and Coenzymes[1]—In separate experiments not shown, the effectiveness of different metal ions and ATP was studied. Mg^{++} (0.003 M) is the most effective. Equimolecular concentrations of Fe^{++} or Fe^{+++} are slightly less effective and the rate is not maintained as well as that with Mg^{++}. Mn^{++} enhances only slightly the action of ATP. Co^{++} is without effect, while Ca^{++} and Cu^{++} completely abolish the ATP

TABLE IV

Effect of EDTA with and without ATP and Mg^{++} on OAA-Inhibited Succinate Oxidation

Data expressed as microliters of oxygen uptake per mg. of N per hour.

Line No.	Additions	10 min.	20 min.	30 min.	40 min.
1	Succinate	790	760	640	510
2	" + OAA	60	70	60	60
3	" + " + EDTA	75	50	80	90
4	" + " + Mg^{++}	200	165	125	150
5	" + " + " + EDTA	420	400	190	185
6	" + " + ATP	270	190	125	90
7	" + " + " + EDTA	165	150	95	60
8	" + " + " + Mg^{++} + EDTA	820	660	590	520

Conditions as in Tables I and II. EDTA used where indicated to give a final concentration of 0.001 M.

effect or, when the addition of the latter ion is delayed (as described above for Ca^{++}), it immediately restores the inhibition. Results of other experiments indicate that adenosine diphosphate is about as effective as ATP; the combination of DPN and Mg^{++} is only slightly more effective than the metal ion used alone, and inosine triphosphate or triphosphopyridine nucleotide used with Mg^{++} decreases somewhat the relief provided by the metal ion alone.

Changes in OAA and Pyruvate Concentrations—The relief of inhibition by ATP and Mg^{++} is not due to an increased rate of decarboxylation or utilization of OAA. Although in a number of experiments these changes

[1] Cytochrome *c* and the various coenzymes used in this study were purchased from the Sigma Chemical Company and were employed without further purification. EDTA (Sequestrene), obtained from the Alrose Chemical Company, was a disodium salt recrystallized three times.

were followed at 10 minute intervals for 1 hour, only the data during the first 20 minutes are given (Table V), as it is during this time that ATP, Mg^{++}, and EDTA exert the greatest effect in relieving the inhibition. The experimental results given are from a Warburg experiment typical of six others in which simultaneous respiration and keto acid determinations were made. It can be seen that ATP and Mg^{++} stimulate the utilization of OAA, an effect that is further enhanced by EDTA. However, if the actual inhibition of succinate oxidation that occurs is compared with the inhibition expected from the amount of OAA present (see Table I), considerable discrepancy exists. When ATP and Mg^{++} are added (Table V,

TABLE V

Changes in Concentrations of OAA and Pyruvate during First 20 Minutes

Line No.	Reaction mixture	10 min.			20 min.		
		OAA concentration $\times 10^{-3}$ M	Per cent inhibition		OAA concentration $\times 10^{-3}$ M	Per cent inhibition	
			Observed	Expected*		Observed	Expected*
1	Succinate + OAA	0.94	95	90	0.72	95	90
2	" + " + ATP + Mg^{++}	0.80	15	90	0.65	60	85
3	" + " + " + " + " + EDTA	0.60	5	85	0.49	5	80

Experiment in Warburg flasks. Conditions as described in Tables I and II. Keto acid separated and estimated as described in the text. Pyruvate concentration ($\times 10^{-3}$ M) found at 10 minutes: Line 1 = 0.21, Line 2 = 0.19, Line 3 = 0.23; at 20 minutes, Line 1 = 0.28, Line 2 = 0.25, Line 3 = 0.15.

* Calculated from the data given in Table I.

Line 2), there is still sufficient OAA in the flasks to produce at least 90 per cent inhibition during the first 10 minutes; yet only 15 per cent inhibition occurs. At 20 minutes, the inhibition is 60 per cent as compared with an expected 85 per cent, at least. EDTA added together with ATP and Mg^{++} gives more striking differences (Line 3). Both at 10 and 20 minutes sufficient OAA is present to produce from 80 to 85 per cent inhibition; yet the rate of succinate oxidation at these intervals is only 5 per cent lower than that in uninhibited succinate controls. It is apparent, then, that enough OAA is present during this time, but it is not in a form that can inhibit the succinoxidase system.

Effect of Preincubating Enzyme—Preincubated enzymes (without added substrates) show little or no capacity to respond to ATP and Mg^{++} (Table VI, Line 3), even though such preparations have a highly active succinoxi-

dase system (Line 1) which is equally as sensitive to OAA as fresh systems (Line 2). In some experiments, preincubation for 20 minutes causes a complete loss; in others, preincubation for 40 minutes results in a 75 per cent loss. The presence of succinate, OAA, or Mg^{++} during the preincubation does little to affect the extent of this loss (not shown). On the other hand, EDTA added before preincubation to give a final concentration of 0.001 M largely prevents this loss (Line 4). This chelating agent is also capable of partly restoring this capacity to aged enzyme preparations when added to the reaction mixture after the preincubation (Line 5). It should also be noted that such EDTA-protected systems are just as sensitive to

TABLE VI

Effect of Preincubating Enzyme at 38°

Data given in microliters of oxygen consumed per mg. of N per hour.

Line No.	Additions during preincubation	Additions after preincubation	10 min.	20 min.	30 min.	40 min.
1	None	Succinate	770	640	570	480
2	"	" + OAA	85	95	55	50
3	"	" + " + ATP + Mg^{++}	215	190	155	140
4	EDTA	" + " + " + "	675	430	305	280
5	None	" + " + " + " + EDTA	490	290	270	270
6	EDTA	Succinate + OAA + ATP + Mg^{++} (Ca^{++} added after 10 min. reading)	700	90	110	125

The enzyme was preincubated in a Dubnoff shaker for 30 minutes and then added to Warburg flasks containing the reaction mixtures indicated above and in the concentrations given in Tables I and II. All other conditions as previously described.

the delayed additions of Ca^{++} (Line 6) described above for fresh preparations.

Similar results, as reported above, are obtained when phosphate instead of tris(hydroxymethyl)aminomethane (Tris) is used as a buffer. Mitochondria give findings similar to those from whole homogenates, provided these particles are prepared in the cold from a saline or sucrose homogenate containing EDTA (0.001 M), washed twice in 0.9 per cent NaCl or sucrose and then resuspended in 0.9 per cent NaCl. Such preparations appear to be able to maintain the ATP-Mg^{++} effect for a longer period than whole homogenates.

DISCUSSION

Pardee and Potter (2) attributed the relief from OAA inhibition to the ATP in their reaction mixture. These results show that either Mg^{++} or

ATP can produce this effect, but it is the combination that is most effective. The inhibitory action of EDTA on ATP when this coenzyme is used without an excess of Mg^{++} suggests that ATP requires a metal ion that can be removed by EDTA. On the other hand, the mechanism of action of EDTA in enhancing the action of a combination of ATP and Mg^{++} can be deduced by some of the known characteristics of the chelating agent and the results reported here. It has been shown (6) that EDTA added to a mixture containing Ca^{++} and Mg^{++} tends to bind more of the Ca^{++} than the Mg^{++}. Since the data presented here, as well as those reported by Pardee and Potter (2), establish that Ca^{++} reverses or prevents this action of ATP and Mg^{++}, it appears that the enhancing effect of EDTA (under conditions in which there is an excess of Mg^{++}) is due to the removal of the Ca^{++} that may be present in the enzyme preparations. Further support for this idea is seen in experiments in which delayed Ca^{++} additions restore the ability of OAA to inhibit succinoxidase despite the presence of ATP, Mg^{++}, and the chelating agent. In these experiments Ca^{++} was added in excess of the amount that could be bound by the EDTA.

The slight changes in the concentrations of the keto acids OAA and pyruvate indicate that relief from inhibition provided by ATP and Mg^{++} is not due to decarboxylation or utilization of the OAA. It appears, therefore, that the OAA must be in a form that is not inhibitory to succinoxidase or that some alteration in the succinoxidase system is produced, rendering it incapable of combining with OAA but still able to oxidize succinate. There is a possibility that ATP and Mg^{++} alter the succinoxidase system in some way and so may affect its affinity for OAA or succinate. The inhibition of succinate oxidation by ATP and Mg^{++} (Table II, Line 3) would seem to support such an assumption. However, it is more likely that this inhibition is due to the increase in keto acids (Line 3) rather than to a direct effect on the enzyme. On the other hand, there is considerable support for the premise that OAA forms metal complexes before it is decarboxylated (7, 8). Recently, it has been suggested that an enzyme (OAA decarboxylase?) also acts as an agent for combining the metal with OAA in such a way as to enhance its activity (9). In this connection, our results with preincubated enzymes are of interest. It appears that, if an "Mg^{++} complex-forming" enzyme is involved, it is easily inactivated, although not irreversibly, by such aging procedures.

On the basis of present information, the phenomenon can be understood best by assuming that Mg^{++} competes with some other metal ion, such as Ca^{++}, for a common site. The action of Ca^{++} in restoring the inhibition, the enhancing effect of EDTA, and even the results obtained with preincubated enzymes are in keeping with this premise. As to the effects of preincubation, it is noteworthy that Slater and Cleland (10) reported that a

loss of activity of α-ketoglutaric oxidase in aged preparations was associated with an increased Ca^{++} concentration in the sarcosomes. They also observed that EDTA prevented a large part of this loss. From these considerations and the above data, it is suggested that ATP, Mg^{++}, and an enzyme are involved in the formation of a metal-enzyme complex with OAA. As a working hypothesis for further study, it is assumed that this initial complex-forming reaction removes OAA from the reaction mixture and so prevents the keto acid from combining with the affinity points of succinic dehydrogenase. This complex is not stable. Ca^{++} and certain other metal ions such as Cu^{++} rapidly cause a dissociation, releasing OAA which is then available to inhibit succinate oxidation. This phenomenon and its significance are the subject of further study in this laboratory.

SUMMARY

1. Adenosine triphosphate or Mg^{++} relieves the inhibition produced by oxalacetate on the succinoxidase system. This effect is reversed by Ca^{++} and enhanced by ethylenediaminetetraacetic acid.

2. The action requires an enzyme that is readily, but reversibly, inactivated by preincubation. EDTA can protect against this inactivation or restore the activity in preincubated preparations.

3. The relief from inhibition produced by adenosine triphosphate and Mg^{++} is not due to a decarboxylation or utilization of oxalacetate. The results are discussed from the point of view of the possibility of a metal complex-forming reaction involving OAA.

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PURIFICATION OF LIVER XANTHINE OXIDASE

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The pioneer investigations of Westerfeld and Richert (1, 2) on a nutritional xanthine oxidase factor led to the observations of De Renzo *et al.* (3, 4) that molybdenum specifically increased the level of intestinal xanthine oxidase. Since then, it has been shown by a number of investigators (5–9) that molybdenum is present in purified milk xanthine oxidase in a 1:2 ratio with flavin. The association of molybdenum with xanthine oxidase has given new significance to this enzyme as a model for electron transport through flavoprotein systems (10). From the work of Mackler *et al.* (8), it appears that molybdenum functions in the xanthine oxidase system in the transfer of electrons from the flavoprotein to 1 electron acceptors. In addition to molybdenum, there is evidence from the recent work of Richert and Westerfeld (9) that iron is also an integral part of xanthine oxidase.

In view of these significant developments in relation to xanthine oxidase it seemed desirable to be able to prepare highly purified xanthine oxidase for study from liver as well as from milk. Until the present, milk has been the only source from which highly purified preparations of xanthine oxidase have been obtained. The purification of liver xanthine oxidase has been carried out to a limited extent in the past by Corran *et al.* (11) and by Richert *et al.* (12). In this paper a procedure is described for the preparation of liver xanthine oxidase with specific activities comparable to those of the most active preparations obtained from milk (Q_{O_2} about 1500). Some properties of the purified enzyme are also described. A preliminary report of this work has been given elsewhere (13).

Materials and Methods

DPNH[1] (90 per cent pure) and cytochrome *c* were products of the Sigma Chemical Company, St. Louis, Missouri. FAD was kindly contributed by Dr. A. Kornberg and highly purified rat liver catalase was a gift from Dr. V. Price and Dr. R. Greenfield of the National Cancer Institute.

Xanthine oxidase activity was measured spectrophotometrically at 24° by following the rate of uric acid formation from the oxidation of xanthine

[1] The abbreviations used in this paper are as follows: DPNH, reduced diphosphopyridine nucleotide; FAD, flavin adenine dinucleotide.

XUM

at 290 mμ according to the method of Kalckar (14), as described by Morell (15), at pH 8.3 in pyrophosphate buffer. A unit of enzyme was defined as the amount causing a change in optical density equivalent to an oxygen consumption of 1 μl. per hour in the absence of catalase. Specific activity is given as Q_{O_2}, microliters of O_2 per hour per mg. of protein. Anaerobic experiments were carried out in Thunberg tubes sealed to 1 cm. absorption cells. The tubes were alternately evacuated and flushed with helium two times. The reduction of cytochrome *c* was determined essentially according to the method of Horecker and Heppel (16).

Protein was estimated by the turbidimetric method of Bücher (17) with soluble liver protein solutions whose protein content had been determined by Kjeldahl nitrogen as standards. FAD was determined fluorometrically by the method of Burch, Bessey, and Lowry (18) by using the fluorometer and photomultiplier attachments of the Beckman model DU spectrophotometer. Molybdenum was determined according to Ellis and Olson (19) and total iron according to Keilin and Hartree (20).

Purification of Liver Xanthine Oxidase

1 kilo of frozen calf liver was homogenized with 750 ml. of 0.05 M phosphate buffer, pH 7.5, in a Waring blendor for 3 minutes at reduced speed to avoid excessive foaming. The thick suspension was quickly stirred into 3.75 liters of the phosphate buffer previously heated to 70°. The temperature of the mixture was brought to 60° over a period of 5 minutes and held there for an additional 5 minutes. The suspension was cooled to 0° with stirring and coagulated proteins were removed by centrifugation (volume of extract = 4 liters, Q_{O_2} = 6.7, total activity = 240,700 units).

To the slightly turbid extract, 1.8 kilos of ammonium sulfate were added and the suspension was allowed to stand in a tall glass cylinder overnight in the cold. Most of the clear red supernatant solution was siphoned off and the remaining suspension was centrifuged at 75,000 × *g* for 30 minutes. The hard packed precipitate (148 ml.) was dissolved in 0.1 M phosphate buffer, pH 7.5, to make a total volume of 600 ml. To this solution 91 gm. of ammonium sulfate were added. The precipitate was removed by centrifugation and discarded. An additional 91 gm. of ammonium sulfate were added to the supernatant solution. The precipitate was centrifuged at 75,000 × *g* and kept in stoppered tubes at 0° for further enzyme purification (Q_{O_2} = 15, total activity = 197,000 units).

A portion of the ammonium sulfate precipitate (Q_{O_2} = 15) containing 98,000 units of enzyme activity was dissolved in 0.1 M phosphate buffer, pH 7.5, to make a total volume of 262 ml. The protein solution was digested with 100 mg. of crude trypsin for 3 hours at 38°. The mixture was heated at 60° for 5 minutes, then cooled to 0° and centrifuged. To 234 ml. of the clear supernatant solution, 37.5 gm. of ammonium sulfate were added.

After standing overnight in the cold, the precipitate was centrifuged and discarded. To 242 ml. of the clarified solution, 36.8 gm. of ammonium sulfate were added. The precipitate was centrifuged at 75,000 × *g* and dissolved in 250 ml. of 0.05 M phosphate buffer, pH 6.2 (Q_{O_2} = 32, total activity = 68,400 units). This solution was about 3 per cent saturated with ammonium sulfate.

The above enzyme solution was passed with gentle suction through a column of calcium phosphate gel on cellulose prepared according to the method of Price and Greenfield (21) modified to contain 3 times more cellulose. The column was prepared with 250 ml. of fresh gel suspension in a 65 mm. sintered glass Büchner funnel. After passage of the enzyme solution, the column was washed with 0.1 M phosphate buffer, pH 6.8, until most of the color was removed from the gel. The gel was discarded and the enzyme solution, together with the wash liquid from the gel treatment, was diluted with an equal volume of water and again passed through a gel column prepared as before. After washing the column with 200 ml. of 0.05 M phosphate buffer, pH 7.5, the enzyme was eluted from the gel with 0.25 M phosphate buffer, pH 7.5, as a yellow-orange band (68 ml.). The enzyme was precipitated from solution by adding ammonium sulfate to 60 per cent saturation (Q_{O_2} = 434, total activity = 38,000 units). The specific activity of this preparation was raised to 685 by repeating the gel treatments with only 10 ml. of gel suspension and proportionately smaller volumes of buffer. The total activity recovered from this step was 16,500 units. The gel adsorption steps were carried out at room temperature, 25°, and required about 5 hours for completion.

The enzyme, after precipitation with ammonium sulfate at 60 per cent saturation, was dissolved in 2 ml. of 0.1 M phosphate buffer, pH 7.5, and treated with 1 ml. of saturated ammonium sulfate. The precipitate was centrifuged out after 1 hour and discarded. To the supernatant solution 0.5 ml. of saturated ammonium sulfate was added and the precipitate was immediately removed by centrifugation (Q_{O_2} = 1360, total activity = 7000 units). A second precipitate was obtained from the supernatant solution by adding 0.25 ml. of saturated ammonium sulfate (Q_{O_2} = 1070, total activity = 5700 units). These two enzyme fractions represent an over-all yield of about 10 per cent of the activity in the original liver extract. By repeating the ammonium sulfate precipitation on the combined fractions a product with a Q_{O_2} of 1500 was obtained between the limits of 33 and 40 per cent saturation in about a 5 per cent over-all yield.

Properties of Purified Liver Xanthine Oxidase

FAD, Molybdenum, and Iron—Analytical data are presented in Table I for the FAD, molybdenum, and iron content of purified liver xanthine oxidase preparations. The results indicate that liver xanthine oxidase con-

tains 1 mole of FAD to 1 atom of molybdenum to 4 atoms of iron. For milk xanthine oxidase this ratio has been reported as 2:1:8, respectively (9). The molybdenum content of liver and milk xanthine oxidase appears to be about equal, 0.03 per cent (7–9), but the FAD and iron content of the liver enzyme corresponds to only one-half of that of the milk enzyme. On account of the lower FAD content of liver xanthine oxidase, the $D_{280m\mu}/D_{450m\mu}$ ratio approached a value of 10 instead of values of 5 to 7 found for milk xanthine oxidase (7, 8, 11, 15). Results on an aged enzyme preparation (Preparation 3) suggest that, as the enzyme lost activity by storage in saturated ammonium sulfate, FAD and molybdenum were lost in a 1:1 ratio, but iron was firmly held. It has been observed that the intensity of the orange-brown color of fresh preparations diminishes as the enzyme ages

TABLE I

FAD, Molybdenum, and Iron Content of Purified Liver Xanthine Oxidase Preparations

Preparation No.	Q_{O_2}	FAD	Molybdenum	Iron	$\frac{FAD}{Mo}$	$\frac{Fe}{Mo}$
		μmoles per gm.	*microatoms per gm.*	*microatoms per gm.*		
1	1550	3.1	3.5	13.4	0.89	3.8
2	1460	2.7	3.2	14.0	0.84	4.4
3*	690*	1.4	1.7	17.1	0.82	9.9

* Original activity, $Q_{O_2} = 960$. The preparation was kept in saturated ammonium sulfate for 8 weeks at 0°. The precipitate was centrifuged and redissolved just prior to analysis.

and loses activity. The addition of FAD or molybdenum and iron compounds to aged or fresh enzyme preparations had no effect on the xanthine oxidase activity measured.

Absorption Spectra—As seen in Fig. 1, the spectrum of oxidized liver xanthine oxidase exhibits a continuously increasing absorption below 400 mμ and a rather broad maximum around 450 mμ. The reduced spectrum shows a generally decreased absorption with dithionite causing somewhat more reduction than xanthine. The difference spectrum (oxidized minus reduced) shows the characteristic flavin peaks at 370 and 450 mμ. These spectral characteristics are not unlike those described for milk xanthine oxidase (8, 15, 16, 22). The slight irregularity noticed between 410 and 420 mμ is probably due to a small amount of heme impurity. A pronounced absorption in this region which was significantly reducible by xanthine was often observed in preparations of high specific activity, but, after careful refractionation with calcium phosphate gel and ammonium sulfate, only a trace of this material remained with the enzyme.

Treatment of the enzyme with excess xanthine reduced the absorption at 450 mμ to the extent of 71 per cent of the reduction given by dithionite. Of the total reduction by xanthine (measured after 1 hour), 67 per cent occurred in the time required to make the first reading, about 30 seconds. Thereafter, the rate of reduction was very slow. Similar observations have been made on milk xanthine oxidase by Morell (15) who ascribed the re-

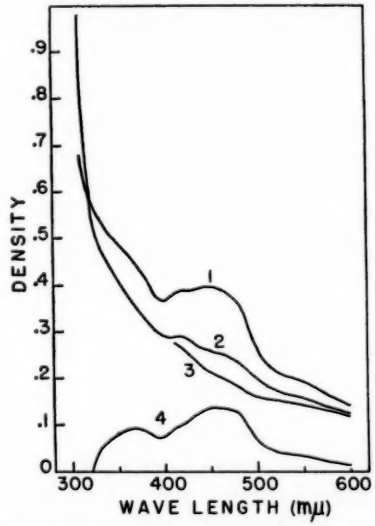

FIG. 1. Absorption spectra of purified liver xanthine oxidase. The Thunberg tube contained 2.7 mg. of enzyme of $Q_{O_2} = 1550$ in 1 ml. of 0.1 M phosphate buffer, pH 7.5. The reduced spectrum was determined anaerobically 1 hour after the addition of 0.35 μmole of xanthine from the stopper. Reduction by dithionite was measured last after opening the tube and adding a few crystals of $Na_2S_2O_4$. Curve 1, oxidized; Curve 2, reduced with xanthine; Curve 3, reduced with dithionite; Curve 4, oxidized minus reduced (with xanthine).

duction behavior to the presence of active and inactive enzyme to which FAD is attached. The observations of Avis *et al.* (7) that crystalline xanthine oxidase preparations varied widely in specific activity lend support to the idea that variable amounts of active and inactive enzyme may be present. Mackler *et al.* (8), however, contend that the partial reduction of enzyme FAD by substrates can be explained on the basis of an oxidation-reduction equilibrium between the enzyme and substrate system.

Substrate Specificity and Electron Acceptors—In the presence of 2,6-dichlorophenolindophenol and under anaerobic conditions, purified liver xanthine oxidase oxidized xanthine, DPNH, and acetaldehyde at decolorization

rates shown in Table II. If the rate of xanthine oxidation is given an arbitrary value of 100, the rates of oxidation of the other substrates are 48 for DPNH and 24 for acetaldehyde. Crude preparations of the enzyme oxidized these substrates at essentially the same relative rates as the purified enzyme. Corran *et al.* (11) reported similar relative rates of oxidation for a partially purified xanthine oxidase preparation from hog liver. In other experiments in this laboratory, similar relative rates of oxidation of these substrates have been observed for xanthine oxidase of mouse and rat liver and mouse hepatoma. A comparison with rates obtained for milk

TABLE II

Effect of DPNH and Acetaldehyde on Xanthine Oxidation

The Thunberg tube contained 1.0 ml. of 0.1 M phosphate buffer, pH 7.5, 0.1 ml. of 1 per cent serum albumin, 0.1 ml. of 0.00125 M 2,6-dichlorophenolindophenol, 0.05 ml. of substrate,* and water to make a final volume of 3.0 ml.; the stopper contained 0.027 mg. of liver xanthine oxidase (Q_{O_2} = 1550) in 0.05 ml. of 0.1 M phosphate buffer, pH 7.5. $\Delta E_{600\ m\mu}$ per minute is the average decolorization rate over the interval 30 seconds to 2½ minutes at 24°.

Substrate	$\Delta E_{600\ m\mu}$ per min.
Xanthine	0.079
DPNH	0.038
CH_3CHO	0.019
Xanthine + CH_3CHO	0.021
" + DPNH	0.109
DPNH + CH_3CHO	0.066

* 0.005 M xanthine, 0.015 M DPNH, 10 M CH_3CHO. DPNH was placed in the stopper alone or with the enzyme. A slow non-enzymatic rate of reduction of the dye given by DPNH but not by xanthine or acetaldehyde was subtracted from experimental values in the figures given above.

xanthine oxidase, however, points up significant differences. Under comparable conditions, milk xanthine oxidase oxidizes DPNH and acetaldehyde at rates approximating or exceeding rates of oxidation of xanthine or hypoxanthine (8, 11).

The effect of DPNH and acetaldehyde on xanthine oxidation at saturation levels of substrate is shown in Table II. It is seen that acetaldehyde markedly interfered with xanthine oxidation, whereas DPNH did not. In the former case the decolorization rate approached that of acetaldehyde alone, suggesting that the enzyme affinity for the aldehyde was so great that xanthine was scarcely oxidized. On the other hand, the decolorization rate observed when both xanthine and DPNH were present was the sum of the individual rates. Furthermore, acetaldehyde had no inhibitory effect on DPNH oxidation, as evidenced also by the additive rate observed. These observations are in line with other evidence obtained with milk

xanthine oxidase (8) that the enzymatic site of oxidation of DPNH is quite distinct from the site of oxidation of xanthine, hypoxanthine, and acetaldehyde.

The rate of xanthine oxidation at pH 7.5 with 2,6-dichlorophenolindophenol as electron acceptor was about the same as that with O_2, of the order of 1 μmole per minute per mg. of enzyme. DPNH, however, was not appreciably oxidized unless the dye was present. In the presence of O_2, acetaldehyde apparently inactivated the enzyme so that dye reduction stopped altogether in a few minutes. Similar observations have been made on the effect of acetaldehyde on milk xanthine oxidase (16, 22).

The reactivity of the liver xanthine oxidase system with 1 electron acceptors appeared to be far less than has been observed with milk xanthine oxidase (8, 15, 16). The rates of cytochrome *c* reduction in the oxidation of xanthine and DPNH by the liver enzyme were of the order of 5×10^{-3} μmole of cytochrome reduced per minute per mg. of enzyme at 24°. No significant change in rate was observed by adding excessive amounts of catalase, molybdenum as molybdic oxide or sodium molybdate, various iron salts, FAD, or combinations of these substances. Cytochrome reduction did not proceed any faster under anaerobic conditions. Ferricyanide and 2,4-dinitrophenol were also only very slowly reduced by liver xanthine oxidase.

DISCUSSION

Xanthine oxidase can be readily isolated in a purified state from calf liver according to the procedure developed without unusual losses, resulting from the instability of the enzyme (11). In a concentrated state after heat treatment at 60°, the crude enzyme loses little activity over a period of several weeks at 0°. The purified enzyme, however, particularly in dilute solutions can lose as much as 50 per cent of its activity in a week under similar conditions. Xanthine oxidase has also been isolated in a similar state of purity and in similar yields from rat liver. The purification achieved can be estimated as approximately 2000-fold on the basis of a Q_{O_2} of 1.5 at 38° for whole rat liver (23) and 1500 at 24° for the purified enzyme.

The properties of purified liver xanthine oxidase studied so far indicate that the enzyme is a molybdoflavoprotein similar to that present in milk, but with some differences. These differences lie in the significantly lower FAD and iron content of liver xanthine oxidase and in the extremely slow rate at which the enzyme catalyzes the reduction of cytochrome *c*, ferricyanide, and 2,4-dinitrophenol. An adequate explanation for the slow reactivity with 1 electron acceptors cannot be offered at the present time; basic differences in certain cofactor associations involving FAD, molybdenum, iron, and possibly other unknown components may exist between liver and milk xanthine oxidase.

SUMMARY

A procedure has been developed for the purification of liver xanthine oxidase with specific activities comparable to those of highly purified milk xanthine oxidase (Q_{O_2} about 1500). The properties of the purified enzyme indicate that it is a molybdoflavoprotein similar to milk xanthine oxidase but containing only one-half as much FAD and iron. Purified liver xanthine oxidase contained 1 mole of FAD to 1 atom of molybdenum to 4 atoms of iron. In the presence of 2,6-dichlorophenolindophenol the liver enzyme oxidized xanthine, DPNH, and acetaldehyde at the following relative rates: 100, 48, and 24, respectively. Acetaldehyde greatly interfered with xanthine oxidation, but DPNH did not. Acetaldehyde did not interfere with DPNH oxidation. Liver xanthine oxidase catalyzed the reduction of cytochrome *c*, ferricyanide, and 2,4-dinitrophenol at extremely slow rates.

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ON THE ORIGIN OF DEOXYRIBOSE IN ESCHERICHIA COLI AND T6r+ BACTERIOPHAGE*

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The problem of the origin of the deoxyribose of nucleic acid has received little attention. Racker (1, 2) has demonstrated the presence of an enzyme in *Escherichia coli* capable of synthesizing deoxyribose-5-phosphate from acetaldehyde and glyceraldehyde-3-phosphate. A similar enzyme has been demonstrated in mammalian muscle (3). However, the low affinity of the enzyme for acetaldehyde, combined with a lack of evidence that free acetaldehyde occurs in more than trace amounts in the two biological materials studied, raises a question of the functional importance of the demonstrated enzymatic capability.

Certain findings in this laboratory were consistent with a mechanism of deoxyribose synthesis that required triose phosphate. *E. coli* contains the enzymes of the phosphogluconate pathway of glucose degradation (4) as well as the enzymes required for glycolysis (5). The proportion of the glucose degraded by each of the two mechanisms is altered from the normal ratio by infection with a virulent bacteriophage (6). Although the rate of glucose uptake is not altered, less of the metabolized glucose is degraded by the oxidative pathway in the infected cell than in the normal cell. There is no change in the total amount of ribose nucleic acid present in the infected cell, nor is there any turnover of that ribose nucleic acid (7, 8). However, deoxyribose nucleic acid synthesis proceeds at a rate approximately 4 times the rate in the normal growing cell (9). If triose phosphate, formed primarily by glycolysis, were required for deoxyribose synthesis, it appeared possible that the observed shift in the manner of glucose degradation was related directly to the acceleration in deoxyribose synthesis.

Other mechanisms of deoxyribose synthesis have been suggested by experimental findings. The hypothesis that polymeric ribose nucleic acid is

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the precursor of deoxyribose nucleic acid does not appear to be true in the fertilized *Arbacia* egg. The inertness of the ribose nucleic acid in infected *E. coli* cells ruled out the suggested conversion in this system also. However, there is evidence to suggest that a conversion of ribose to deoxyribose can occur at the nucleoside or nucleotide level. Thus, although rats do not incorporate N^{15}-labeled uracil or cytosine into the somatic nucleic acids, administration of N^{15}-labeled uridine and cytidine resulted in both labeled ribose nucleic acid and labeled deoxyribose nucleic acid (10). This has been interpreted as evidence that a unit larger than the free pyrimidine, although not necessarily the complete ribonucleoside, may serve as precursor of both ribose nucleic acid and deoxyribose nucleic acid. Another report in the literature, if substantiated, would give more convincing evidence for the conversion of the sugar moiety of the ribonucleoside to deoxyribose. Rose and Schweigert (11) report the result of a single experiment based on a procedure whereby it was possible to determine the ratio of the activity of a nucleoside sugar moiety alone to the activity of the entire nucleoside molecule. This ratio was obtained both for the uniformly C^{14}-labeled cytidine administered to rats and for the cytidine and deoxycytidine of the isolated ribose nucleic acid and deoxyribose nucleic acid of the sacrificed animals. These ratios were all of the same order, indicating that the intact ribonucleoside had served as a precursor of deoxycytidine units.

The suggestion that a secondary hydroxyl group may be biologically reduced to a methene group, as required in the conversion of ribose to deoxyribose, is not without precedent. The formation of an acyl coenzyme A compound from a hydroxyacyl coenzyme A compound is a recently elucidated example, involving primary dehydration, with subsequent reduction of the resulting unsaturated compound (12). These are the same steps involved in the conversion of 3-phosphoglycerate to lactate. In addition it has been shown that *Pseudomonas saccharophila* contains an enzyme system for the conversion of 6-phosphogluconate to pyruvate and 3-phosphoglycerate, and an intermediate compound in this reaction sequence has been identified as 2-keto-3-deoxy-6-phosphogluconate (13).

In this paper we present evidence that the enzymatic condensation of acetaldehyde and glyceraldehyde-3-phosphate does not appear to be the sole functioning mechanism of deoxyribose synthesis in aerobically growing cells of *E. coli*. It is suggested that a second mechanism is also functioning in the growth of these cells. On the other hand, in the case of virus production, our evidence is consistent with the hypothesis that a major route of deoxyribose synthesis for phage nucleic acid may well involve the triose phosphate and acetaldehyde mechanism.

Methods

Growth of Bacteria—The method used in the aerobic growth of the bacterial cells in a synthetic medium supplemented with glucose has been described previously (14). To a suspension of washed cells, trichloroacetic acid was added to give a concentration of 5 per cent, and the resulting precipitate was washed with alcohol and ether. This dried material was subjected to an alkaline extraction of the ribose nucleic acid by the procedure of Schmidt and Thannhauser (15). Deoxyribose nucleic acid was obtained from the residue by the method of Ogur and Rosen (16).

Infection of E. coli with Bacteriophage; Isolation of T6r$^+$ Virus—Prior to infection, *E. coli* was grown aerobically in the synthetic medium supplemented with glucose as described previously; growth was halted in the logarithmic phase by chilling, and the cells were sedimented by centrifugation. These cells were washed by suspension in the salt medium and again sedimented. Finally, they were suspended at a titer of 2×10^8 in the synthetic medium supplemented with 1.2 mg. per ml. of glucose-1-C^{14} and 50 γ per ml. of DL-tryptophan, and within 1 minute were infected with a purified preparation of T6r$^+$ virus at a virus to cell ratio of 5. The culture was aerated during the subsequent latent period; there is no visible lysis of the bacterial cells during the first 3 hours, and a period of 6 to 7 hours is required for maximal lysis of the culture. The total quantity of deoxyribose nucleic acid present increases linearly during the first 3 hours, resulting in a 10- to 12-fold increase, and decreases only slightly after that time (17). However, of this total deoxyribose nucleic acid only about 25 per cent is subsequently isolated as purified phage after lysis, owing to the losses by absorption to cellular débris and to incomplete sedimentation in the cycles of low and high speed centrifugation required in the isolation of the phage. Nevertheless, it has been shown that the only type of deoxyribose present at the point of maximal deoxyribose nucleic acid concentration is that type characteristic of the virus (18), and it is reasonable to assume that the isolated deoxyribose acid is representative of the total deoxyribose nucleic acid synthesized.

Conversion of Nucleic Acid Deoxyribose to Levulinic Acid—An empirical set of conditions, suggested by the work of Levene and Mori (19), was used. Deoxyribose nucleic acid-containing extracts were neutralized with KOH, chilled, and freed of the precipitated $KClO_4$ by centrifugation; suspensions of phage in saline were used directly. To the deoxyribose nucleic acid solution, one-tenth its volume of concentrated sulfuric acid was added. This digestion mixture was heated in a ground glass-stoppered cylinder inserted in an oil bath maintained at 130° in a position such that the liquid surface within the cylinder was level with the surface of the heating bath.

The stopper was momentarily loosened after immersion of the cylinder before being securely wired in place. It was essential that the cylinder should be no more than one-third to one-half full, as the upper portion of the vessel acts as a condenser. At the end of 5 hours the cylinder was removed from the bath and cooled before opening. If the cylinder was properly stoppered during the heating, no concentration occurred, and the solution was a light brown color.

Preparation and Isolation of 2,4-Dinitrophenylhydrazone of Levulinic Acid—Levulinic acid was separated from the sulfuric acid digestion mixture by continuous extraction with ether. To the ether solution 1 ml. of water was added for each 6 μmoles of deoxyribose nucleic acid phosphorus originally present; the ether was evaporated from the aqueous levulinic acid solution by directing a stream of air on its surface. A reagent solution containing 12 mg. of 2,4-dinitrophenylhydrazine per ml. of "15 per cent" perchloric acid, or 60 μmoles of reagent per ml., was used. An equal volume of this reagent was added to the levulinic acid solution and the mixture heated 30 minutes on a boiling water bath. After immediate cooling, the solution was extracted with ether in a continuous extractor. The ether solution was concentrated without use of heat to less than 10 ml. in volume and washed with 2.5 ml. portions of a 0.5 per cent ammonium carbonate solution. As long as this wash had an acidic pH, it was discarded; when the pH became alkaline, the aqueous layer was transferred to a volumetric flask. Three alkaline washes were usually sufficient to extract all the levulinic acid derivative.

The ultraviolet absorption spectrum of levulinic acid 2,4-dinitrophenylhydrazone in ammonium carbonate solution is indicated in Fig. 1. A quantitative measurement was obtained on the alkaline extracts by determining the absorption at 3775 A and applying a correction for the absorption of a reagent blank carried through the preparative procedure simultaneously.

The levulinic acid derivative was purified by partition chromatography. Ether-washed Celite 535 (Johns-Manville) was used as inert support; the aqueous phase, presaturated with the organic phase, was a buffer made by dissolving 0.2 equivalent of Tris (tris(hydroxymethyl)aminomethane) and 0.1 equivalent of boric acid in 100 ml. of water and adjusting the pH to 8.90 by addition of a few ml. of a 2 N Tris solution, as necessary. In the preparation of the column 0.6 ml. of this aqueous phase was added per gm. of Celite, and the mixture was stirred to a homogeneous dry powder. This was suspended in ethyl acetate, presaturated with the aqueous phase, and poured into a column containing a supported pad of glass wool. The Celite was evenly dispersed and packed firmly with a plunger and the top

of the column protected with a pad of glass wool. Just before using, this column was washed out with ether, presaturated with buffer.

The ammonium carbonate solution of levulinic acid derivative was freed of salt by evaporation to dryness. This crude dried derivative may be partially extracted with ether directly; the remainder was dissolved in a total of 0.9 ml. of water, used in several portions, and added to 1.5 gm. of silicic acid suspended in anhydrous ether. An even suspension of the silica gel was obtained by stirring and then poured into a column plugged with glass wool. The filtrate from this column, with ether washings as nec-

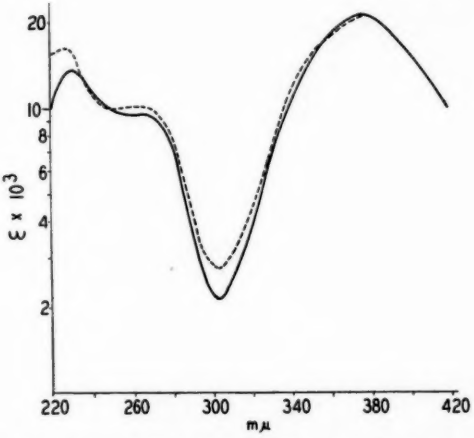

FIG. 1. Absorption of levulinic acid 2,4-dinitrophenylhydrazone; dash line, derivative of *E. coli* deoxyribose nucleic acid.

essary, was combined with the direct ether extract of the derivative and concentrated to a small volume for application to the partition column. The column was developed with ether, free of peroxides, and presaturated with Tris buffer.

The crude derivative separated on the column into five fractions; the second component eluted was shown to be levulinic acid 2,4-dinitrophenylhydrazone by its absorption spectrum. The ether solution was washed free of traces of buffer by several treatments with water saturated with CO_2 and concentrated to approximately one-tenth its initial volume with an air stream. The aqueous layer that separated during this concentration was removed, and the ether solution was finally evaporated to dryness with an inert gas. When the quantity was sufficient, this material could be crystallized from ethanol. Material so obtained from the deoxyribose nucleic

acid of *E. coli* had the typical crystal habit (acicular, rhombic) and the melting point of an authentic levulinic acid derivative (207°), and, in mixed melting point determinations, showed no depression. The ultraviolet absorption of this material is presented in Fig. 1.

Radioactivity Determinations—The D-glucose-1-C^{14} used in these experiments was obtained through the courtesy of Dr. H. Isbell of the National Bureau of Standards. The aqueous solutions used in the experiments were diluted and quantities of the order of 0.3 to 0.5 μmole were spread directly on aluminum planchets and counted with a windowless gas flow counter flushed with a helium-isobutane mixture. The 2,4-dinitrophenylhydrazone derivative of levulinic acid was dissolved in peroxide-free dioxane prior to plating. Since the density of material on the planchets did not exceed 0.15 mg. per sq. cm., no corrections were made for self-absorption.

TABLE I

Experiments with Normal, Aerobically Growing Cells of E. coli

Experiment No.	Glucose-1-C^{14} added to growth medium	Deoxyribose derivative	$\frac{\text{Deoxyribose activity}}{\text{Glucose activity}}$
	c.p.m. per μmole	*c.p.m. per μmole*	*per cent*
XI	2018	409	20.3
XX	1709	423	24.8
XXI	1709	448	26.2
XXVI	1299	258	19.8
XXVII	1299	305	23.5

Results

Deoxyribose Synthesized in Growing Cells—The results obtained in experiments in which *E. coli* was grown in a synthetic medium containing glucose-1-C^{14} as sole carbon source are listed in Table I. It is seen that the deoxyribose of the growing cells has 20 to 26 per cent of the molar specific activity of the glucose-1-C^{14} available to the cells.

Utilization of Host Constituents for Phage Deoxyribose Nucleic Acid Synthesis—Estimates have been made of the use of host cell material, in contrast to use of the constituents of the suspending medium, for phage synthesis after infection. Studies with P^{32} have indicated that 14 to 26 per cent of the deoxyribose nucleic acid phosphorus of the phage was present in the host cell at the time of infection (7, 20), largely as deoxyribose nucleic acid (21). A study in which the nucleic acid bases of the host cell were labeled showed that 8 to 12 per cent of the pyrimidines subsequently isolated from the virus deoxyribose nucleic acid was of host origin (22, 23) and 15 to 30 per cent of the purines (24). None of these studies, of course, gives specific information on the use of preformed deoxyribose in the syn-

thesis of phage deoxyribose nucleic acid. An attempt was made to obtain a definite answer to this question by the infection of host cells grown in the presence of glucose-1-C^{14}, but washed and resuspended in a non-isotopic medium at the time of infection. The molar specific activity of the deoxyribose of the cells at the time of infection was determined. The following results were obtained:

Deoxyribose nucleic acid derivative, cells before infection	281	c.p.m. per μmole
" " " " of phage	64	" " "
Activity of host deoxyribose recovered in phage deoxyribose	23	%

It would seem, therefore, that 23 per cent of the deoxyribose isolated as phage deoxyribose nucleic acid may be formed from material present in the

TABLE II

Phage Produced by E. coli in Glucose-1-C^{14} Medium

Experiment No.	Glucose-1-C^{14} of infection medium	Deoxyribose derivative		Corrected deoxyribose activity / Glucose activity
		Observed activity	Corrected activity*	
	c.p.m. per μmole	*c.p.m. per μmole*	*c.p.m. per μmole*	*per cent*
XIII	2059	668	868	42.1
XV	1852	836	1086	58.7
XXII	1452	454	590	40.6
XXIII	1452	617	802	55.2
XXIV	1435	654	850	58.6
XXV	1435	468	608	42.3

* × 1.3; see the text.

host cell prior to infection, a result within the range of values obtained for the host deoxyribose nucleic acid phosphorus and base contributions. It may be inferred that nucleotide units resulting from the degradation of host deoxyribose nucleic acid are available for resynthesis. A correction factor of $1/(1 - 0.23)$, or 1.30, may reasonably be applied to the observed molar specific activities obtained for deoxyribose in the infection experiments.

Deoxyribose Synthesized by Infected Cells—Table II presents results obtained when phage was produced by non-labeled cells suspended in a medium containing glucose-1-C^{14} at the time they were infected with phage. It is seen that the deoxyribose synthesized under these conditions is 41 to 59 per cent as active as the glucose of the medium. This value is approximately twice that obtained in the experiments with normal, growing cells.

DISCUSSION

Cells of *E. coli* have been shown to have the enzymatic ability to condense acetaldehyde with glyceraldehyde-3-phosphate. If it is assumed

that this represents the functioning mechanism of deoxyribose biosynthesis in this organism, it would be necessary to postulate that when labeled glucose is the sole carbon source available to the cells the deoxypentose formed would have a molar specific activity equal to the sum of the respective activities of the two precursor compounds. These may be predicted from the value of the equilibrated triose phosphate pool, since the only known precursor of acetaldehyde in biological material is represented by the α- and β-carbon atoms of pyruvate, a compound derived from the triose phosphate pool. Glyceraldehyde-3-phosphate is, of course, one of the molecular species of the pool itself.

When glucose-1-C^{14} is the only carbon compound available to an organism, all the hexose phosphates may be expected to have equal molar specific activities, in each case due to activity localized solely in the 1 position. If it is assumed that the only source of triose is the aldolase cleavage of fructose diphosphate, a maximal value is obtained for the activity of the equilibrated triose phosphate pool; it would have a molar specific activity one-half as great as the molar specific activity of the hexose compounds. However, it has been estimated that as much as 38 per cent of the glucose metabolized by *E. coli* may be degraded by the phosphogluconate pathway, by which each glucose-1-C^{14} molecule may form 1 molecule of non-isotopic triose phosphate, while at least 62 per cent is degraded by the glycolytic scheme by which each molecule of labeled glucose gives rise to 1 inactive and 1 radioactive triose phosphate molecule. Thus $62/(38 + (2 \times 62))$ of the triose phosphate molecules formed will have the molar specific activity of the glucose, and the equilibrated pool thus has a molar specific activity of 38 per cent of that of the reference glucose. This is the minimal possible figure.

Thus the triose phosphate pool, and therefore the glyceraldehyde-3-phosphate, will have a molar specific activity of 38 to 50 per cent of that of the reference glucose. Also, if we postulate that acetaldehyde arises from triose phosphate via pyruvate, it too would have a molar specific activity of 38 to 50 per cent. Deoxyribose-5-phosphate synthesized by a combination of acetaldehyde and glyceraldehyde-3-phosphate would therefore have an activity of 76 to 100 per cent of that of the reference glucose.

The molar specific activity values obtained for deoxyribose in our experiments with glucose-1-C^{14} are of the order of 20 to 26 per cent of that of the reference glucose. It is therefore obvious that the functioning biosynthetic mechanism is not correctly represented by the enzymatic condensation that has been demonstrated *in vitro*.

A different source of acetaldehyde might be considered that could give rise to a non-isotopic molecule. It has been shown by many workers that

certain microorganisms may convert the C-1 and C-2 atoms of pentoses into acetate (25, 26); recently it has been reported that in the rat ribose-1-C^{14} is an excellent donor of radioactive acetyl groups (27). It is logical to assume that conversion of the pentose to free acetaldehyde is therefore possible, and, in view of the origin of ribose in this organism (14), formation of acetaldehyde of very low activity might thus be visualized. Nevertheless, the observed activity of deoxyribose remains significantly lower than the value estimated for the glyceraldehyde-3-phosphate alone, and it would seem that there must be a second mechanism functioning in the biosynthesis of this pentose in *E. coli*.

The pathway of glucose degradation utilized by cells of *P. saccharophila* is of interest in this connection. As recently shown, 6-phosphogluconate may be split non-oxidatively to triose phosphate plus pyruvate. Although the steps of this pathway are not yet clarified, 2-keto-3-deoxy-6-phosphogluconate has been identified as an intermediate compound. The chemical relationship of this compound to deoxyribose is obvious. Preliminary studies have shown that cells of *E. coli*, under certain conditions, have the ability to degrade 6-phosphogluconate in a non-oxidative manner.[1] The question of the existence of this type of glucose degradation in aerobic cells of *E. coli* utilizing glucose as sole carbon source is unsettled at the present time; if present, it does not appear to be a very active system. Deoxyribose synthesized from glucose-1-C^{14} by this "*Pseudomonas* mechanism" would of course be unlabeled.

The possibility of a conversion of ribose to deoxyribose as suggested by the studies on the incorporation of ribonucleosides into deoxyribose nucleic acid remains another possibility for an alternative mechanism for deoxyribose biosynthesis. We have shown previously that ribose synthesized by cells of *E. coli* during aerobic growth on glucose-1-C^{14} has a molar specific activity of the same order as that now found for the deoxyribose.

The infected *E. coli* cell has an altered metabolism. Our studies on deoxyribose synthesis demonstrate another facet of this change. In contrast to the findings in the normal growing cell, deoxyribose that is synthesized for incorporation into bacteriophage has 41 to 59 per cent of the molar specific activity of the glucose present in the medium during the latent period. It is therefore possible that in these cells a compound of the equilibrated triose phosphate pool may be incorporated into the deoxyribose molecules. We suggest that use of a second, alternative path of deoxyribose synthesis is eliminated or curtailed in the infected cell. It is known that, in comparison to normal cells, less of the degraded glucose-1-C^{14} is handled in a manner such as to liberate preferentially the C-1 atom as

[1] Scott, D. B. McN., personal communication.

CO_2. A non-oxidative decarboxylation of 2-keto-3-deoxy-6-phosphogluconate as a step in deoxyribose synthesis would require the formation of CO_2 from the C-1 atom of that compound. The oxidative decarboxylation of 6-phosphogluconate leading to ribose formation as a step in deoxyribose synthesis would also result in the liberation of the C-1 atom as CO_2. Of course, the possible functioning of either of these hypothetical biosynthetic mechanisms cannot be evaluated from the experimental findings.

SUMMARY

1. A method is described for the isolation, in a pure state, of a characteristic derivative of the deoxyribose of bacterial deoxyribonucleic acid.

2. Deoxyribose synthesized by *Escherichia coli* growing aerobically in a synthetic medium containing glucose-1-C^{14} as sole carbon source has 20 to 26 per cent the molar specific activity of the glucose available to the cells. The enzymatic condensation of acetaldehyde and glyceraldehyde-3-phosphate does not appear to be the sole functioning mechanism of deoxyribose biosynthesis in aerobically growing cells of *E. coli*.

3. The deoxyribose of bacteriophage, synthesized by cells of *E. coli* from glucose-1-C^{14} as the sole carbon source, has 41 to 59 per cent of the molar specific activity of the glucose of the medium. A change in the mechanism utilized for the biosynthesis of deoxyribose may be added to the other known alterations in metabolism caused by infection of *E. coli* with a virulent phage.

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THE BIOSYNTHESIS OF ISOLEUCINE AND VALINE

II. INDEPENDENCE OF THE BIOSYNTHETIC PATHWAYS IN NEUROSPORA*

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Paper I of this series (1) described the preparation of cell-free extracts from *Escherichia coli* and *Neurospora crassa* which catalyze the dehydration of the dihydroxy acid precursors of isoleucine and valine (DHI and DHV) to the keto acid precursors (KI and KV). The present paper is concerned with the origin of the dihydroxy acids in *Neurospora*.

On the basis of nutritional experiments with induced mutant strains of microorganisms, it has been generally accepted that isoleucine is formed biosynthetically from a 4-carbon precursor metabolically related to homoserine and threonine (2, 3). The origin of valine, on the other hand, was completely obscure until recent findings pointed to pyruvate as the chief source of valine carbon in yeast (4) and in *E. coli* (5, 6). In 1951, Tatum and Adelberg (7) proposed a common biosynthetic origin for isoleucine and valine from a hypothetical 6-carbon dicarboxylic acid. The latter was presumed to arise by condensation of acetate with a 4-carbon unit related to threonine. Their hypothesis was offered as explanation for the striking similarity in distribution of C^{14} found in DHI and DHV when a *Neurospora* strain was fed either methyl-labeled or carboxyl-labeled acetate.

While their hypothesis was consistent with their isotope data, the possibility remained that isoleucine and valine are formed by separate pathways in *Neurospora*. These alternative views are diagramed in Fig. 1, which also shows the locations of the metabolic blocks in the organisms used for experimental test. The experiments reported below were designed to distinguish between these alternative hypotheses. *Neurospora* strain 16117 (*iv-1*) was genetically combined with strain 35423 (*thr*), a mutant

* Supported by a contract between the Office of Naval Research and the Regents of the University of California.

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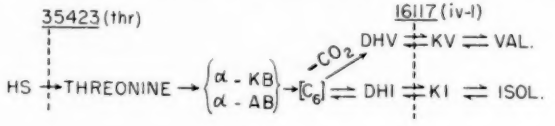
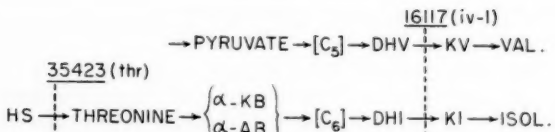
which requires L-threonine for growth. Washed mycelial pads of the double mutant and of the single mutant (strain 16117) were then compared for DHI and DHV accumulation in the presence and in the absence of L-threonine and related compounds.

According to Scheme I (Fig. 1), introduction of the L-threonine requirement into strain 16117 by recombination with strain 35423 should greatly reduce the accumulation of *both* dihydroxy acids, and both accumulations should be specifically stimulated by threonine, α-aminobutyrate, or α-keto-



Scheme I



Scheme II

FIG. 1. Alternative schemes for the biosynthesis of isoleucine and valine. Threonine is shown to be directly on the pathway to isoleucine, although this has been disputed (8). Scheme I depicts the "common origin hypothesis" proposed by Tatum and Adelberg (7); Scheme II indicates independent origins as established in yeast (4) and in *E. coli* (5, 6). HS = homoserine; α-KB = α-ketobutyric acid; α-AB = α-aminobutyric acid; DHV, DHI = dihydroxy acids corresponding to valine and isoleucine, respectively; KV, KI = keto acids corresponding to valine and isoleucine, respectively.

butyrate. Scheme II, on the other hand, predicts that only DHI production should be so affected. The quantities of dihydroxy acids accumulated actually provide a measure of their synthesis, since strain 16117 lacks the dehydrase system converting them to keto acids (1). Our present experiments show that the predicted changes in DHI accumulation are *not* paralleled by changes in accumulation of DHV; therefore a common origin for valine and isoleucine in *Neurospora* can now be ruled out.

Materials and Methods

Organisms—Strains 35423 (threonine-requiring), designated *thr*, and 16117 (isoleucine-valine requiring), designated *iv-1*, of *N. crassa* were used in this investigation. Reisolated strain 16117-1-1 was used for the cross

with strain 35423, since this accumulates large amounts of DHI and DHV. The double mutant strain (*iv-1, thr*) was obtained from an ascus containing four wild type and four double mutant spores and was used for the experiments presented in Table II. Further proof of the inferred genotype of this strain was provided from the results of a back-cross to wild type strain SY7A. Asci from the back-cross were dissected serially and the three expected types of asci were obtained: *iv-1 thr*, wild type; *iv-1*, *thr*, *iv-1 thr*, wild type; and *iv-1*, *thr*. From among these progeny there were chosen at random seven single mutant strains (*iv-1*) and seven double mutant strains (*iv-1 thr*), for use in the experiment presented in Table I.

Preparation of Mycelial Pads—Conidia obtained from 4 day-old slants were inoculated into 125 ml. Erlenmeyer flasks containing 50 ml. of minimal medium (9) supplemented with 4 mg. of DL-isoleucine and 3 mg. of DL-valine. In the case of strain *iv-1 thr*, the medium also contained 2 mg. of L-threonine. The sucrose content of the growth medium was 1.0 per cent. Sucrose was autoclaved separately. The flasks were incubated at 30° for 4 days without shaking, followed by 24 hours on a rotary shaker. Controls lacking each amino acid were included in every experiment. The absence of growth in the controls indicated that the cultures were genetically stable for the mutant characters in question.

The growth medium was decanted from the 5 day-old cultures and replaced with 10 ml. of minimal medium lacking sucrose and amino acids. The pads were washed and starved by shaking 10 hours in this medium at 30°, following which the wash liquid was decanted and replaced with fresh minimal medium containing experimental supplements.

Dihydroxy Acid Determinations—Aliquots of 0.1 or 0.2 ml. were taken at specified times and applied to Whatman No. 1 filter paper. The samples were chromatographed and the dihydroxy acid spots eluted and determined as described previously (10).

Results

Dihydroxy Acid Accumulation in Minimal Medium—A number of strains of *iv-1* and of *iv-1 thr* were obtained from a single cross as described in "Materials and methods." Dihydroxy acid accumulation in minimal medium by duplicate mycelial pads of seven strains of each type was determined (Table I). When the data for each group were averaged, it was found that the *iv-1 thr* strains accumulated only 36 per cent[1] as much DHI as did the *iv-1* strains, but 88 per cent as much DHV. The data were subjected to a statistical analysis of variance within and between the two

[1] The fact that the double mutant can still produce a small amount of DHI agrees with the fact that the parent strain 35423 grows slowly in minimal medium and thus appears to synthesize threonine at a limiting rate.

TABLE I

Accumulation of Dihydroxy Acids in Minimal Medium by Neurospora Single Mutant iv-1 and Double Mutant iv-1 thr

Group No.	Strain iv-1		Strain iv-1 thr	
	DHI	DHV	DHI	DHV
	μmoles	*μmoles*	*μmoles*	*μmoles*
1	12.6	21.6	6.9	27.4
2	8.4	18.4	3.0	12.2
3	10.1	19.5	6.1	18.6
4	16.4	16.8	3.0	4.4
5	14.8	21.7	2.4	15.6
6	11.7	23.0	4.2	18.3
7	12.9	21.6	6.2	29.0
Average.....	12.4	20.4	4.5	17.9

The values are for 24 hour accumulations in minimal medium containing 0.4 per cent sucrose; each figure is an average for duplicate mycelial pads. See the section "Materials and methods" for the procedures.

TABLE II

Stimulation of Dihydroxy Acid Production in Neurospora Double Mutant Strain iv-1 thr

Compound added to minimal medium	Increase over control	
	DHI	DHV
	μmoles	*μmoles*
L-Threonine	31.5	9.7
α-Ketobutyric acid	39.6	3.7
DL-α-Aminobutyric acid	46.8	17.5
DL-Homoserine	2.8	Inhibition
L-Aspartic acid	Inhibition	"
L-Glutamic acid	4.3	13.0
Succinic acid	6.0	11.5
DL-Lactic acid	2.5	15.1
Glycerol	2.6	8.2
Formic acid	Inhibition	5.3
D-Glucose	3.8	19.5
Sucrose	2.6	3.7
L-Serine	0.0	26.4
Glycine	5.5	27.9

The medium contained 0.34 per cent sucrose in addition to the supplements listed. Each compound was supplied as 400 microatoms of carbon (*e.g.*, 100 μmoles of C_4 compounds, 200 μmoles of C_2 compounds, etc.). The values are for the increase over unsupplemented controls, determined after 40 hours incubation. See "Materials and methods" for the basal medium and procedures. In most cases the figure represents an average value for two to four separate cultures.

mutant classes (11). A null hypothesis was employed, and the variance ratio, F, was calculated for each dihydroxy acid. The results indicated that the two mutant classes had behaved as members of a single normal population with respect to DHV production. The F value based on DHI accumulations, however, was significant at only 0.1 per cent. The difference between the amounts of DHI accumulated by the two mutant groups is thus highly significant.

Effect of L-*Threonine and Other Compounds on Dihydroxy Acid Accumulation*—Dihydroxy acid accumulation by the original culture of strain *iv-1 thr* was tested in the presence of various compounds (Table II). Since most of these compounds are utilizable as carbon sources by *Neurospora*, each compound was supplied at a concentration which yielded the same amount of carbon (400 microatoms). The results show that DHI production is greatly stimulated by L-threonine, α-aminobutyric acid, and α-ketobutyric acid, but not by any other compound tested. In contrast, the accumulation of DHV does not follow this pattern, but is stimulated to some extent by most of the compounds supplied, particularly by serine and glycine.

When similar experiments were carried out with strain *iv-1*, the results were similar, except that homoserine significantly stimulated DHI production.

DISCUSSION

Since these experiments were begun, the origin of valine from pyruvic acid rather than from a 4-carbon precursor of isoleucine has been clearly established for *E. coli* and for yeast by the work of Abelson (5), Cohen and Hirsch (6), and Strassman *et al.* (4). The results reported in this paper show that in *Neurospora*, also, isoleucine and valine have an independent origin. The high activity of L-serine and glycine in stimulating DHV production probably reflects a rôle of pyruvate in valine biosynthesis by *Neurospora*, since this organism possesses an active L-serine deaminase (12) and appears to interconvert serine and glycine (13).

The conclusion that isoleucine, but not valine, can derive 4 of its carbon atoms from threonine has been confirmed by the isotopic tracer experiments reported in Paper III of this series (14).

SUMMARY

1. *Neurospora* mutant 16117, which accumulates the dihydroxy acid precursors of isoleucine and valine (DHI and DHV), was genetically combined with a strain which specifically requires L-threonine for growth. Introduction of the second block into strain 16117 caused a marked decrease in DHI production, which could be restored to high levels by the

addition of L-threonine, α-aminobutyrate, or α-ketobutyrate, but not by a variety of other compounds.

2. Accumulation of DHV by the double mutant was not significantly different from that of strain 16117 and was not specifically stimulated by L-threonine or related compounds. The view that valine and isoleucine are formed from a common 4-carbon intermediate in *Neurospora* is thus no longer tenable. Of the compounds tested for stimulation of DHV production, serine and glycine were the most effective, presumably as a result of their conversion to pyruvic acid.

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THE BIOSYNTHESIS OF ISOLEUCINE AND VALINE

III. TRACER EXPERIMENTS WITH L-THREONINE*

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In Paper II of this series (1) it was reported that the resting mycelium of a *Neurospora* double mutant strain excreted up to 35 μmoles of the dihydroxy acid precursor of isoleucine (DHI)[1] when fed 100 μmoles of L-threonine. These results could be interpreted to mean either that threonine is convertible to DHI or that threonine indirectly stimulates endogenous DHI production. If the former relationship holds, there is then the question of which of the 6 carbon atoms of DHI can be derived from threonine. Accordingly, tracer experiments with C^{14}-L-threonine and isotopic competition experiments (2) with C^{14}-acetate and unlabeled L-threonine were conducted. The DHI formed was degraded and the contribution of threonine to each position determined. Preliminary accounts of this work have appeared elsewhere (3, 4).

Materials and Methods

Organisms—Two strains of *Neurospora crassa* were used. Strain 16117 is an induced mutant strain requiring isoleucine and valine for growth in minimal medium and accumulates the dihydroxy acids corresponding to isoleucine and valine (5, 6). Strain 35423-16117 is a genetically combined double mutant, combining strain 16117 with strain 35423, which has a highly specific growth requirement for L-threonine. The double mutant strain was prepared by Dr. R. W. Barratt, as described in Paper II (1); it requires all three amino acids for growth.

Preparation of Mycelial Pads—Cultures were grown in supplemented minimal medium (7) and washed mycelial pads prepared as described in Paper II (1). Pads of strain 16117 had an average dry weight of 106 mg. and of strain 35423-16117 an average dry weight of 124 mg.

Isolation of DHI and DHV—The mycelial pads were removed from the incubation mixtures by filtration, and the filtrate was continuously extracted for 24 hours with peroxide-free ether, first at pH 8.3 and then at pH

* Supported by a contract between the Office of Naval Research and the Regents of the University of California.

[1] The following abbreviations will be used: DHI represents the dihydroxy acid corresponding to isoleucine; DHV the dihydroxy acid corresponding to valine.

2.0. The final ether extract was evaporated on the steam bath, leaving an aqueous residue which was applied in a narrow band to a sheet of Whatman No. 1 filter paper with the aid of a kymograph. The sheet was developed with 70:30 ether-benzene saturated with water and made 3 M with formic acid, by ascending chromatography. After drying in air at room temperature, the dihydroxy acids were located by scanning with a Geiger-Müller tube or by spraying a narrow strip with bromophenol blue in ethanol, and the corresponding bands were cut from the sheet. The paper strips were eluted by the capillary descent of water from a wick; the eluates were neutralized, and aliquots were assayed for dihydroxy acid content (8) and for radioactivity.

Degradation of DHI—The radioactive DHI was diluted with a known amount of unlabeled carrier material and degraded to CO_2 (carbons 1 and 2),[2] iodoform (carbon 4), and propionic acid (carbons 3, 5, and 6) by the procedure of Tatum and Adelberg (9). The propionic acid was purified by chromatography on silica gel columns (10), with 3 per cent butanol in chloroform. The eluate fraction containing the major acid band was extracted with a known volume of 0.02 N sodium hydroxide and the amount of acid determined by back-titration. The identity of the acid as propionic was verified by comparison with known propionic acid on paper chromatograms (11).

The sodium propionate was evaporated to dryness in a shell vial and quantitatively decarboxylated by the procedure of Katz, Abraham, and Chaikoff (12). Carbon 3 was thus obtained as barium carbonate; carbons 5 and 6 remained as ethylamine sulfate in the reaction mixture. The ethylamine sulfate was quantitatively oxidized to 2 moles of CO_2 as follows: The reaction mixture in the shell vial was washed into the outer compartment of a diffusion vessel and neutralized to pH 6. 1 gm. of potassium persulfate was added, followed by 1.0 ml. of aqueous 4 per cent silver nitrate. A shell vial containing 0.5 ml. of exactly 1 N NaOH was placed in the center well; the flask was then capped, evacuated, and heated to 80° in the oven for 1 hour. After cooling and releasing the vacuum, the vial was removed and the carbonate precipitated with an excess of barium chloride. Yields were determined by back-titration to phenolphthalein, following which the barium carbonate was centrifuged, washed with water and with alcohol, and resuspended in alcohol for plating and counting of radioactivity.

Measurement of Radioactivity—All samples were evaporated on tared copper planchets, weighed, and counted with an assembly consisting of an end-window Geiger-Müller tube and a Berkeley decimal scaler. All samples were counted to within 3 per cent standard error and corrected for self-absorption.

[2] See Fig. 1 for the numbering system.

Chemicals—Carrier (unlabeled) DHI was isolated from filtrates of *Neurospora* strain 16117, crystallized repeatedly as the quinine salt (13), recovered as the free acid by ion exchange, and titrated with standard NaOH. 1,2-C^{14}-DL-Threonine was the gift of Dr. David M. Greenberg; 1-C^{14}-acetate was supplied by Dr. W. Dauben; 2-C^{14}-acetate was purchased from Tracerlab, Inc.

Results

Conversion of 1,2-C^{14}-Threonine to DHI—A washed mycelial pad of *Neurospora* strain 35423-16117 was suspended in 10 ml. of minimal medium containing 100 μmoles of sucrose, 100 μmoles of unlabeled L-threonine, and 2 μmoles of 1,2-C^{14}-DL-threonine containing 120,000 c.p.m. The L-threonine thus had a final specific activity of 600 c.p.m. per μmole.[3] The flask was shaken at 30° for 40 hours; the DHI and DHV which accumulated were then isolated and purified and their radioactivities determined. The results are presented in Table I. The DHI was found to have 73 per cent of the specific activity of the L-threonine supplied, and 98 per cent of the activity was in the carboxyl and α-carbon atoms; this showed the derivation of carbon atoms 1 and 2 of DHI from carbon atoms 1 and 2 of threonine. The DHV contained negligible activity.

The specific activity of the DHI indicated a 27 per cent dilution of the labeled threonine. In Paper II (1), it was shown that combining strain 16117 with strain 35423 caused a reduction in endogenous DHI production to 36 per cent of normal. Both the tracer experiment and the accumulation experiment suggest that strain 35423-16117 synthesizes threonine at roughly one-third of the normal rate, in agreement with the observation that strain 35423 itself is able to grow slowly in minimal medium without exogenous threonine.

Isotopic Competition—In order to determine the fate of carbon atoms 3 and 4 of threonine, isotopic competition experiments were conducted. In this type of experiment, a product is formed from a radioactive carbon source in the presence of a large pool of unlabeled metabolite. If the metabolite is an intermediate in the biosynthesis of the product, it will suppress incorporation of radioactivity into that product either by simple dilution or by "competition" (2).

Two resting mycelial pads of *Neurospora* strain 16117 were allowed to produce DHI from sucrose and C^{14}-labeled acetate; one of the two flasks also contained 100 μmoles of unlabeled L-threonine. 2-C^{14}-Acetate was used in Experiment 1 and 1,2-C^{14}-acetate in Experiment 2. The DHI formed in each case was isolated and degraded. The results are presented in Fig. 1 and Table II. Unlabeled L-threonine suppressed the incorpora-

[3] D-Threonine has no growth-stimulating activity for strain 35423 and is considered inert in this experiment.

tion of radioactivity into DHI to the extent of 69 per cent in Experiment 1 and 83 per cent in Experiment 2. As shown in Fig. 1, the incorporation of radioactivity was suppressed in carbon atoms 1 and 2 and, surprisingly, in carbon atoms 5 and 6. The activities in carbon atoms 3 and 4 were actually higher than in the controls, probably reflecting physiological variance between the two *Neurospora* mycelia.

TABLE I

Radioactivities of DHI and DHV Produced by N. crassa Fed 1,2-C¹⁴-Threonine

	DHI	DHV
	c.p.m. per μmole	*c.p.m. per μmole*
Whole molecule	436	22
Carboxyl and α-carbons	410	
Remainder of molecule*	6	

Organism fed L-threonine containing 600 c.p.m. per μmole. See "Materials and methods" for details.

* Degradation product prepared as the 2,4-dinitrophenylhydrazone of methyl ethyl ketone.

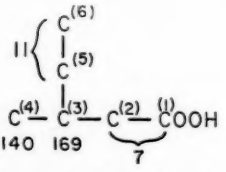


FIG. 1. Effect of L-threonine on incorporation of 2-C¹⁴-acetate into DHI by *N. crassa*. The carbon skeleton of DHI is shown; the numbering system is in parentheses. The large numbers indicate the radioactivity incorporated in the presence of unlabeled L-threonine, expressed as per cent of the control (no threonine added). See Table II for explanation.

Two separate experiments are reported in Table II. In Experiment 1, 2-C¹⁴-acetate was used; while this permits conclusions to be drawn regarding positions 2, 3, 4, and 5 of isoleucine (see derivation of isoleucine from acetate, diagramed in the "Discussion") the effect of threonine on position 6 is less clearly established, since the acetate methyl group contributes little radioactivity to this carbon (9). Accordingly, Experiment 2 was carried out with 1,2-C¹⁴-acetate, and the results compared with those of Experiment 1. In Experiment 1, threonine caused a reduction of radioactivity of carbon atoms 3, 5, and 6 to 44 per cent of the control level (Line *f*, Table II); most of the radioactivity was in positions 3 and 5. In Experiment 2, with 1,2-C¹⁴-acetate, the level of radioactivity in positions 3, 5, and

6 in the control (no threonine added) was 3 times as high as in Experiment 1, as a result of the contribution of acetate carboxyl to position 6; nevertheless, the presence of threonine lowered the radioactivity to the same level as that found in Experiment 1 (Line *g*, Table II). It is thus clear that threonine completely suppressed the incorporation of radioactivity into position 6.

TABLE II

Effect of L-*Threonine on Incorporation of Radioactivity into DHI by Resting Mycelia of N. crassa*

Line	Carbon atoms	Degradation product or basis for calculation	Experiment 1		Experiment 2		Effect of L-threonine; C^{14} per cent of control	
			L-Threonine present	No L-threonine (control)	L-Threonine present	No L-threonine (control)	Experiment 1	Experiment 2
a	1, 2	$BaCO_3$	148	2160	314	3360	7	9
b	3	"	405	240			169	
c	4	CHI_3	482	345	441	410	140	110
d	5, 6	$BaCO_3$	99	910			11	
e	3, 4, 5, 6	MEK-2,4-DNPH*			900	3610		25
f	3, 5, 6	(*b*) + (*d*)	504	1150			44	
g	3, 5, 6	(*e*) − (*c*)			459	3200		14
h	All	(*a*) + (*b*) + (*c*) + (*d*)	1134	3655			31	
j	"	(*a*) + (*e*)			1214	6970		17

In Experiment 1, washed mycelial pads suspended in 10 ml. of minimal medium containing 100 μmoles of sucrose and 1.05 mg. of 2-C^{14}-sodium acetate, with an activity of 1.21×10^6 c.p.m.; with and without 100 μmoles of unlabeled L-threonine. In Experiment 2, the same, except that 1.03 mg. of 1,2-C^{14}-sodium acetate were present, containing 1.57×10^6 c.p.m., instead of 1-C^{14}-acetate.

* 2,4-Dinitrophenylhydrazone of methyl ethyl ketone.

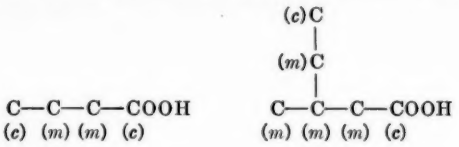
Experiment 2 also confirms Experiment 1 concerning the effect of threonine on incorporation of radioactivity into positions 1, 2, and 4. The data of Experiment 1 are summarized in Fig. 1.

DISCUSSION

The experiments reported here show that exogenous L-threonine does not act as a precursor of DHV, but can supply 4 of the 6 carbon atoms of DHI in *Neurospora*. Furthermore, the 4 carbons do not appear as a continuous sequence in DHI. This finding was foreshadowed by the data of Ehrensvärd *et al.* (14),[4] who found that in various microorganisms supplied

[4] Ehrensvärd, personal communication.

with isotopically labeled acetate the carbons of threonine and of isoleucine are derived as shown in the accompanying diagram. It can be seen that



Threonine Isoleucine

(*m*) = acetate methyl; (*c*) = acetate carboxyl.

there is no sequence of carbon atoms in isoleucine possessing the derivation pattern of the 4 carbon atoms of threonine; our results are completely compatible with the derivations shown.

From the extensive work of Ehrensvärd's group, it is apparent that threonine and isoleucine are unique in possessing a terminal methyl group derivable from the carboxyl group of acetate. Our present experiment shows that this is not a coincidence, but rather reflects the close biosynthetic relationship between threonine and isoleucine.

In considering possible mechanisms to account for the observed distribution of the carbon atoms of threonine in DHI, several facts must be taken into account. Nutritional evidence (15) points to conversion of threonine to α-ketobutyric acid as the first step. Furthermore, the experiments of Ehrensvärd *et al.* show that carbon atoms 3 and 4 of isoleucine have the same derivation as the α- and β-carbons of alanine and serine, and thus are probably equivalent to a decarboxylation product of pyruvic acid. It is attractive to postulate either an aldol or a ketol condensation of α-ketobutyric acid with "active acetaldehyde" to form a 6-carbon intermediate, which would then undergo a pinacol type of rearrangement to give the isoleucine carbon skeleton. Such a mechanism has been proposed to account for a similar phenomenon in valine biosynthesis (16) and has been discussed in detail elsewhere (3, 4, 17). In any case, the mechanism of biosynthesis of DHV and DHI proposed earlier (15) is now untenable.

The author is indebted to Dr. David M. Greenberg for the gift of 1,2-C^{14}-threonine, to Dr. R. W. Barratt for preparation of the double mutant, to Dr. S. Abraham and Dr. J. Katz for advice concerning the degradation of propionic acid, and to Mr. John W. Myers for isolation and purification of the unlabeled dihydroxy acids.

SUMMARY

1. When a *Neurospora* strain which accumulates the dihydroxy acid precursors of isoleucine and valine (DHI and DHV) is fed 1,2-C^{14}-thre-

onine, the DHI formed has C^{14} in the carboxyl and α-carbons with 73 per cent of the specific activity of the radioactive L-threonine, while the DHV has negligible activity.

2. When a similar *Neurospora* strain is used in isotopic competition experiments, L-threonine suppresses the incorporation of radioactivity from C^{14}-labeled acetate into positions 1, 2, 5, and 6 of the isoleucine precursor. An intramolecular rearrangement of a 6-carbon intermediate is proposed to account for these findings.

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METABOLISM OF EPICHOLESTEROL-4-C^{14} IN THE RAT*

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Recent studies on the catabolism of cholesterol-4-C^{14} in the rat have shown that the isotopic carbon is eliminated from the body almost entirely with the feces and that up to 90 per cent of the fecal C^{14} enters the intestine via the bile. Not more than 10 per cent of the C^{14} is excreted directly through the intestinal wall. Approximately 90 per cent of the C^{14} recovered in bile is in the form of bile acids, whereas the remainder consists of non-saponifiable steroids. Three of the bile acids have now been identified as taurocholic, taurochenodeoxycholic, and (tauro?) lithocholic acids (1–5).

The present report deals with the fate of C^{14}-labeled epicholesterol in the rat. This compound is of interest because it differs from cholesterol only in the steric configuration of the OH group on carbon 3. In cholesterol this group has a β configuration, but in epicholesterol it is in the α position, which is also characteristic of most of the naturally occurring bile acids. The possibility was, therefore, considered that epicholesterol is an intermediate in the conversion of cholesterol to bile acids.

EXPERIMENTAL

Epicholesterol-4-C^{14}—The preparation of this labeled steroid has been described (6). Since radioactive steroids deteriorate on standing (7), the epicholesterol-4-C^{14} was purified immediately before use. β-Steroids were removed by precipitation with digitonin, and the supernatant solution was evaporated. The residue was dissolved in pyridine, and the free digitonin was precipitated with ethyl ether and removed by centrifugation. The supernatant solution was evaporated, and the epicholesterol residue was purified by chromatographing it twice on an alumina column (Merck). The epicholesterol was applied to the column in hexane and eluted with 15 per cent ethyl ether in hexane. The melting point of the eluted material was 140°.

Unlabeled epicholesterol was synthesized (8), and its purity was established by melting point and infra-red spectroscopy.

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Taurochenodeoxycholic acid was isolated from goose bile (9). After several reprecipitations from a mixture of methanol and ethyl ether, the acid was chromatographically pure and was identical with synthetic taurochenodeoxycholic acid. Hydrolysis with alkali gave chenodeoxycholic acid, m.p. 137–139°, chromatographically identical with authentic chenodeoxycholic acid.

Treatment of Animals—Male rats of the Long-Evans strain, weighing 200 to 300 gm., were lightly anesthetized with ether. Cannulas were inserted into the common bile ducts (1). The rats received by injection into the tail vein 1 mg. of epicholesterol (specific activity, 3.4×10^6 c.p.m. per mg.) which was prepared for injection as follows: It was dissolved in ethyl ether, and to this solution were added 1 drop of Tween 20 and 0.5 ml. of 0.9 per cent NaCl solution. The ether was removed by evaporation on a steam bath; a clear emulsion remained. The animals were placed in restraining cages and allowed free access to food and water. Bile was collected continuously for 48 hours, the collection intervals ranging from 2 to 10 hours. Feces excreted during the entire period were combined for analysis.

Fractionation Procedures—Aliquots of *bile* (generally 50 μl.) were mounted directly on aluminum disks, and C^{14} was determined in a gas flow counter to ±5 per cent. Mass correction was negligible.

The fractionation of the bile is outlined in Diagram 1. 1 ml. was added to 3 ml. of 4 N NaOH, and the mixture was autoclaved for 4 hours at 120°. After cooling, the mixture was acidified with H_2SO_4 and extracted with several portions of petroleum ether. Since radioactivity in the fatty acids has previously been shown to be negligible (2), the radioactivity found in this petroleum ether extract was attributed to neutral steroids. Further fractionation into α- and β-steroids was accomplished by precipitation with digitonin.

The aqueous phase (referred to as PE-insoluble in Diagram 1) was extracted three times with ethyl ether to remove bile acids. Aliquots of the ether extract were mounted, and their C^{14} content was determined.

Feces were extracted with ethyl alcohol until all radioactivity was removed. Suitable aliquots were mounted, and the C^{14} content was determined. The alcohol extract was saponified and extracted as described above. The neutral steroids were examined in the manner described for bile.

Liver was hydrolyzed with dilute sodium hydroxide, and the neutral steroids were isolated by extraction with petroleum ether. The petroleum ether extract was washed several times with aqueous ethanol and evaporated to dryness. The subsequent treatment of the residue is described below.

Filter Paper Chromatography of Bile—Whole bile samples were chromatographed on Whatman No. 1 filter paper strips as described in detail elsewhere (3). The solvents used were (*a*) collidine-water, (*b*) butanol saturated with water in an ammonia atmosphere, and (*c*) *n*-hexanol-butylamine-water, 100:45:16. The compounds on the paper were located both

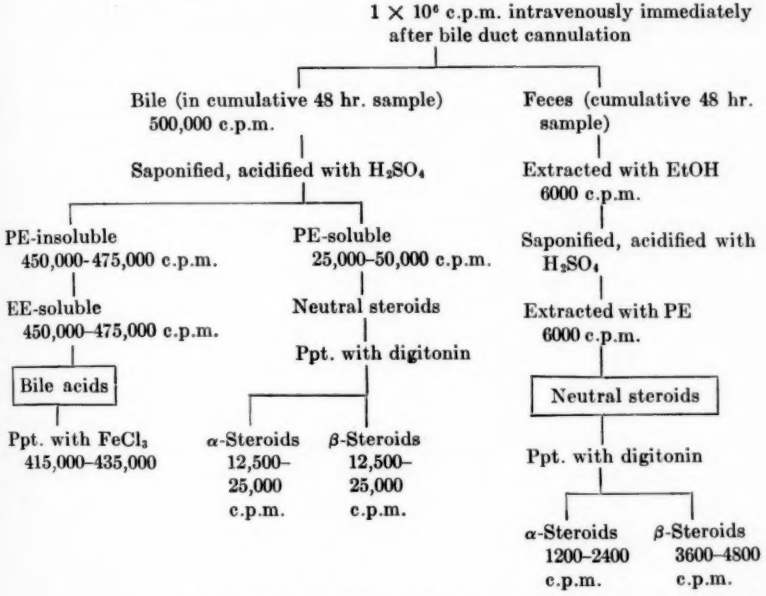

DIAGRAM 1. Distribution of C^{14} in bile and feces accumulated during the 48 hour period after injection of an arbitrarily chosen value of 1 × 10⁶ c.p.m. of epicholesterol. The actual counts injected are given in the experimental section. PE, petroleum ether; EE, ethyl ether; EtOH, ethyl alcohol.

by spraying with antimony trichloride and by preparing radioautographs from the chromatograms.

Results

Recovery of C^{14} in Liver, Bile, and Feces

By the end of 48 hours, approximately 50 per cent of the injected C^{14} was recovered in the bile (Fig. 1). In the same period no more than 0.6 per cent was recovered in the feces. Most of the remaining C^{14} was found in the liver.

Liver—The neutral steroids of the liver were isolated as described above.

About 90 per cent of the C^{14} in this fraction is precipitable with digitonin. Since cholesterol is the major neutral steroid in liver, it was of interest to determine whether C^{14} had been incorporated into cholesterol. The digitonides were therefore dissolved in pyridine, the digitonin was removed by precipitation with ethyl ether, and the supernatant solution was evaporated. The cholesterol was precipitated as the dibromide, as described by Schwenk *et al.* (10). Several recrystallizations of the dibromide (from

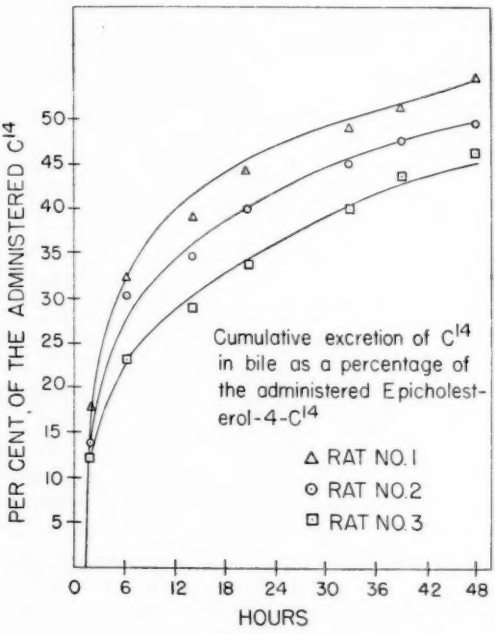

FIG. 1

ethanol, methanol, and aqueous acetone) yielded a product virtually free of C^{14}, thereby indicating that epicholesterol is not converted to cholesterol.

C^{14}-Epicholesterol is, however, converted to a neutral sterol that is precipitable with digitonin and that does not form an insoluble dibromide. This observation suggested that the sterol might be dihydrocholesterol. The following experiments demonstrate that epicholesterol is, indeed, converted to dihydrocholesterol in the liver. The supernatant solution from the bromination was diluted with ethyl ether and washed thoroughly, first with a dilute solution of sodium bisulfite, to remove excess bromine,

and then with water. The ether phase was evaporated, excess carrier dihydrocholesterol was added to the residue, and the mixture was recrystallized successively from ethanol, aqueous acetone, and methanol. No difference in the specific activities of the initial and final products was observed. Two derivatives were then prepared. Part of the dihydrocholesterol was oxidized to cholestanone, m.p. 131° (11), and the remainder was acetylated with pyridine-acetic anhydride (m.p. of acetate, 110°). The corrected specific activities of the two derivatives were identical with those of the initial product. It would therefore appear that dihydrocholesterol is the major *neutral* sterol formed from epicholesterol in the liver and accounts for at least 90 per cent of the digitonin-precipitable C^{14}.

Bile and Feces—The distribution of C^{14} among the various fractions prepared from bile and feces is shown in Diagram 1. For convenience of presentation, the injected dose has been assigned an arbitrary value of 10^6 c.p.m. Not more than 10 per cent of the total C^{14} found in saponified bile was recovered in the petroleum ether-soluble fraction (referred to as the neutral steroid fraction). The C^{14} in this fraction was about equally distributed between digitonin-precipitable and non-precipitable steroids. Carrier cholesterol was added to the digitonin-precipitable fraction, and the β-sterols were analyzed as described above for liver. Again, no C^{14} was found in cholesterol, and the major C^{14}-β-sterol was identified as dihydrocholesterol.

The remaining 90 per cent of the C^{14} present in the hydrolyzed bile was extractable with ether, and practically all of the ether-extractable material was precipitated with $FeCl_3$ by the method of Doubilet (12). Thus it would appear that bile acids constitute the major excretion product of epicholesterol.

The C^{14} found in saponified extract of feces was completely extracted with petroleum ether. About two-thirds of the C^{14} was precipitated with digitonin, but again no C^{14} was found in cholesterol.

Bile Acid End-Products of Epicholesterol Metabolism

To identify the acidic biliary products of epicholesterol metabolism the bile was chromatographed as described above. The positions of the color bands brought out by antimony trichloride were compared with those of the radioactive bands as revealed by radioautography.

Color Bands—Fig. 2, *A* is a diagram of a typical chromatogram of rat bile developed in the collidine-water system. The identification of some of the various color bands was dealt with in a previous communication (3). In descending order, the bands are cholesterol and other neutral steroids (R_F 0.98 to 1), taurochenodeoxycholic acid together with small amounts of (tauro?) lithocholic acid (R_F 0.82), and taurocholic acid (R_F 0.73).

Radioactive Bands—Radioautographs of bile samples obtained at different times after the injection of epicholesterol-4-C^{14} are presented in Fig. 2, *B* to *D*. In the bile sample obtained during the first 2 hours, four C^{14} bands are visible (Fig. 2, *B*). To identify these bands the following experiments were carried out.

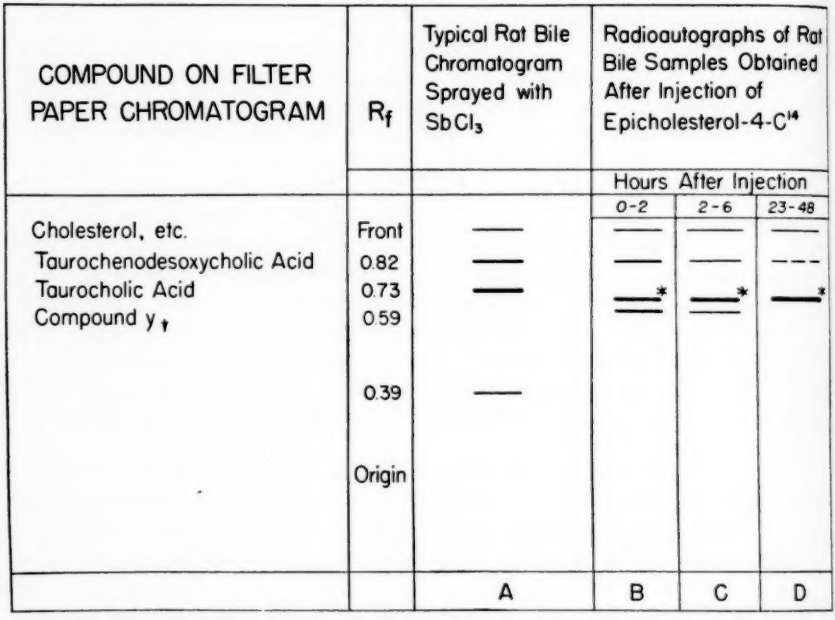

FIG. 2. Chromatogram and radioautographs of rat bile after injection of epicholesterol-4-C^{14}. (*), as pointed out in the text, the position of this compound overlaps, but does not coincide with, that of taurocholic acid. For purposes of presentation this displacement has been exaggerated. (†), Compound Y was observed in our earlier experiments with cholesterol-4-C^{14} (3). A similarly placed band has been observed in the present experiments with epicholesterol-4-C^{14}, but it should not be inferred that these two bands are necessarily identical.

First C^{14} Band—The band at the front corresponds to the position of neutral steroids, such as cholesterol and dihydrocholesterol.

Second C^{14} Band (R_f 0.82)—Superimposition of the films upon the filter paper chromatograms indicated, for all three solvents used, that this C^{14} band corresponded exactly to the color band of taurochenodeoxycholic acid. This suggested that taurochenodeoxycholic acid is a product of epicholesterol metabolism, a suggestion which proved incorrect on further study. A sample of bile collected during the first 12 hours after injection of the epicholesterol was hydrolyzed with 2 N NaOH and acidified, and the

bile acids were fractionated by the method of Bergström and Sjövall (13). Two large radioactive peaks were obtained, the first corresponding to the band at R_F 0.72 and the second to the band at R_F 0.82. Excess chenodeoxycholic acid was added to a sample of the *second peak*, and the mixture was then recrystallized six times from the following solvents: (1) ethyl acetate and petroleum ether, (2) chloroform and petroleum ether, and (3) benzene and methanol. The specific activity of the product obtained after six recrystallizations did not differ from that of the starting material. The final product was then acetylated with pyridine-acetic anhydride on a steam bath for 3 hours, and the acetate was recrystallized from the following solvents: aqueous ethanol, aqueous methanol, and aqueous acetone. After six recrystallizations, the product was virtually free of radioactivity. It must therefore be concluded that the band at R_F 0.82 does not represent chenodeoxycholic acid. By a similar procedure, it was established that the C^{14} compound under consideration is not deoxycholic acid. The chromatographic behavior of this substance suggests, however, that it is the taurine conjugate of an unidentified bile acid possessing two hydroxyl groups (3).

Third C^{14} Band (R_F 0.72)—Superimposition of the films upon the filter paper chromatograms revealed that the C^{14} band overlapped the color band, but they did not coincide. Indeed, good separation of the two bands was achieved by using the butanol-water and hexanol-butylamine systems. Further evidence that the C^{14} substance at R_F 0.72 is not identical with taurocholic acid was obtained by crystallization experiments. A sample of bile collected during the first 12 hours after injection of C^{14}-epicholesterol was hydrolyzed, and the free bile acids were fractionated by column chromatography as described in the preceding section. A sample of the *first* peak eluted from the column by the technique of Bergström and Sjövall was mixed with carrier cholic acid, and the mixture was recrystallized from ethyl acetate. Several recrystallizations sufficed to remove all but a small fraction of the initial radioactivity. Thus, while some of the radioactivity in this band may be due to taurocholic acid, the major C^{14} component is neither taurocholic nor any other conjugate of cholic acid. The position of this band on the paper suggests that the compound responsible is the taurine conjugate of a compound very similar to cholic acid, perhaps an isomer (3).

Fourth C^{14} Band—The C^{14} band at R_F 0.59 has not been identified. It should be recalled that the chromatography of bile from rats injected with cholesterol-4-C^{14} (3) also revealed a C^{14} band in this position (Compound Y), but we have not been able to decide whether the radioactive compound at R_F 0.59 is the same in both cases.

As the excretory process is followed for longer periods, radioactivity fades from the band at R_F 0.82, while the C^{14} band at R_F 0.72 becomes more

prominent (Fig. 2, *C*, 2 to 6 hours). Eventually this band becomes the predominant radioactive excretion product of epicholesterol-4-C^{14}, accompanied by a faint band at R_F 0.82 (Fig. 2, *D*, 23 to 48 hours).

DISCUSSION

It is shown here that the metabolic fate of epicholesterol resembles that of cholesterol in that the major biliary excretion products are bile acids. A study of the radioactive bile acids found in bile after the intravenous injection of cholesterol-4-C^{14} and epicholesterol-4-C^{14} reveals, however, that the biliary end-products of these two isomeric steroids are not identical. Thus, in the case of the former, taurochenodeoxycholic acid is the major C^{14} bile acid excreted in bile during the early intervals and taurocholic, the major acid during the later intervals. In the case of epicholesterol, two unidentified bile acids are encountered, possessing R_F values close to those of taurochenodeoxycholic acid (R_F 0.82) and taurocholic acid (R_F 0.73), respectively, but not identical with them. These unidentified bile acids appear together during the early intervals, whereas in the later intervals the unidentified acid at R_F 0.72 becomes the major biliary product. On the basis of their chromatographic behavior, it is suggested that these unidentified acids are conjugated with taurine and possess structures resembling those of taurochenodeoxycholic and taurocholic acids, respectively.

The metabolism of cholesterol and epicholesterol also differs in other respects. Thus, it has been observed that the C^{14} of intracardially injected epicholesterol-4-C^{14} is excreted in bile about twice as rapidly as is the C^{14} of cholesterol-4-C^{14}. This difference was not observed after intravenous injection and is not readily explained at present. It may also be recalled that the rate of absorption of epicholesterol in the rat is much lower than that of cholesterol (14).

The structural relations of cholesterol, epicholesterol, and the bile acids suggested the possibility that epicholesterol might be an intermediate in the degradation of cholesterol to cholic acid. The observation that epicholesterol is not converted to either taurocholic or taurochenodeoxycholic acid renders this unlikely. The fact that similar compounds are formed, however, suggests a high degree of stereochemical specificity in the metabolic degradation of steroids.

Under our experimental conditions, epicholesterol was not converted to cholesterol by the rat. Instead, dihydrocholesterol appears to be the major neutral sterol recovered from cholesterol both in liver and in bile. In view of the well established conversion of Δ^4-cholestenone to dihydrocholesterol (15, 16), it is conceivable that Δ^4-cholestenone is an intermediate in the conversion of epicholesterol to dihydrocholesterol.

We are indebted to Dr. W. G. Dauben and Dr. J. F. Eastham for the preparation of epicholesterol-4-C^{14}, to Dr. S. Abraham for the preparation of unlabeled epicholesterol, and to Dr. M. D. Siperstein and Mr. H. H. Hernandez for many helpful discussions.

SUMMARY

1. The elimination of the C^{14} of intravenously injected epicholesterol-4-C^{14} from the bodies of rats with cannulated bile ducts has been studied. In the course of 48 hours, approximately 50 per cent of the injected C^{14} was excreted via the bile. In that period, only 0.6 per cent was excreted via the feces. 90 per cent of the biliary C^{14} was in the form of bile acids and the rest in non-saponifiable steroids.

2. The C^{14} bile acids formed resembled taurochenodeoxycholic acid and taurocholic acid, but were not identical with them. It is concluded that epicholesterol is not an intermediate in the conversion of cholesterol to bile acids.

3. Dihydrocholesterol has been shown to be a product of epicholesterol metabolism in the rat.

4. The conversion of epicholesterol to cholesterol could not be demonstrated.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

October, 1905–October, 1955

On the fiftieth anniversary of the founding of this Journal it seems appropriate to review its history and to assess its contribution to the development of our branch of science.

At the turn of the century biochemistry hardly existed as an independent discipline. Organic chemists had for many years been studying the properties and constitution of carbon compounds elaborated by living organisms, but their attention to the chemical processes involved in the formation, function and fate of such compounds was more speculative than experimental. As the need for accurate knowledge of the chemistry of vital processes for the interpretation of physiological observations became increasingly acute, investigators in the medical sciences sporadically engaged in chemical research or, more frequently, enlisted the active interest of specialists in chemistry.

This was the situation when, in 1905, Christian A. Herter and John J. Abel, the professors of pharmacology in the respective medical schools of Columbia and Johns Hopkins Universities, conceived and implemented the establishment of this Journal, and assumed the responsibility of editing it. They were soon joined by A. Newton Richards as assistant editor. In 1909, after five volumes had been published, Abel relinquished his active editorial duties in order to devote all of his available time to the inauguration and editorial management of the Journal of Pharmacology and Experimental Therapeutics. Herter, who appears to have from the outset personally defrayed the considerable deficit incident to publication, died in 1910; continued financial support for the Journal was provided by a group of his friends in the form of the Christian A. Herter Memorial Fund (now part of the capital structure of the American Society of Biological Chemists), the income from which is devoted exclusively to the operation of the Journal.

During the ensuing three years the editors were Henry D. Dakin, Edward K. Dunham and Lafayette B. Mendel, with A. N. Richards as managing editor; in 1914 this group was joined by Donald D. Van Slyke, who shortly thereafter took over the managerial duties from Richards. From 1914 until 1920 the Editorial Board of five underwent no further change; the Journal was, during the years 1914–1925, published from the Rockefeller Institute for Medical Research. In 1920 Dunham resigned, his place being taken by Stanley R. Benedict, and Richards moved from the Board to the Editorial Committee.

In 1925 the publication and management were transferred to the Ameri-

can Society of Biological Chemists, the Council of which reappointed the existing Editorial Committee, setting up in addition a group of Directors and a Finance Committee. The chairman of the Editorial Committee was Otto Folin, who served in that capacity until 1931, when he was succeeded by Philip A. Shaffer. The chairman of the Finance Committee was H. D. Dakin, who served until 1941 and under whose careful supervision the finances of the Journal remained stable and the Herter Memorial Fund underwent a steady growth.

At the time of the transfer of the responsibility of publication to the Society, Van Slyke relinquished the onerous duties of managing editor, which were undertaken by Benedict. To assist him in the details of publication, he installed in the editorial office Miss Mary Smalley, who has remained at her post for nearly thirty years. To her unremitting care and efficiency the Journal owes its long tradition of high standards of publishing technique.

In 1930 Dakin resigned from the Board and his place was taken by Rudolph J. Anderson; in 1933 the number of editors was increased from four to six by the addition of W. Mansfield Clark and Elmer V. McCollum. On the death of Benedict in 1936, Anderson assumed his present duties of managing editor, and the editorial offices were installed in the two top floors of Sterling Tower in New Haven, made available to the Journal through the generosity of Yale University.

Since that time the amount of work handled by the editorial office has grown steadily, and the membership of the Editorial Board has now increased to twenty-seven. With the increased number of essential technical assistants in the editorial office, the congestion in Sterling Tower gradually became unbearable; Yale University has recently installed the office in more commodious quarters on Trumbull Street in New Haven.

As biological chemistry involves the application of any branch of pure chemistry to problems concerned with vital processes, the range of subject matter appropriate to the Journal is inevitably wide. During the first few years of publication, which corresponded chronologically to the infancy of biochemical science, this situation presented little difficulty; papers on analytical, physical, organic and enzyme chemistry, on physiology, pharmacology, nutrition and immunology could be accommodated within the frame of reference of the Journal. Moreover, as at that time there was relatively little demand for space, it was possible to publish comfortably extended—indeed discursive—articles.

At the outset, whenever the number of printed pages reached about 500 a new volume was begun, no attempt being made to insure regularity of issuance. During the first thirty years 8, 15, 21, 22, 23, and 22 volumes

appeared respectively in each successive five year period. In 1938 it was decided that the annual number of volumes be held to five. It was also decided that each volume should contain about 800 pages, but during the ensuing ten years the number of acceptable manuscripts submitted increased so greatly that in the absence of apparently unattainable editorial safeguards the number of pages per volume nearly doubled. In 1949, when the Journal was faced with an alarming deficit, the Editorial Board resolved that six volumes should be published annually, that the 800 page limit should not be grossly exceeded, and that authors should meet the costs of reprints and illustrations.

The restriction of output imposed by these policies, partially offset by an increase in the number of words per page, had to be achieved by even greater editorial insistence on economy of presentation and rigor in the selection of papers for acceptance. These last two editorial functions require time and effort far in excess of the capacity of any individual; the load is accordingly distributed among the members of the Editorial Board, each of whom takes the responsibility for action on manuscripts assigned to him. However, when acceptance is not recommended, confirmation of this decision is invariably secured from at least one other editor before the action becomes final. In order to avoid possible imputation of prejudice, no editor is asked to recommend action on manuscripts emanating from his own laboratory.

In 1939, in view of the great activity in biochemical research, the publication of Letters to the Editors, which should facilitate the rapid dissemination of new information, was instituted. The question whether the advantages gained by this form of publication balanced the disadvantages was problematical from the outset, and after ten years the Society voted its discontinuation.

The prime purpose of the founders of the Journal was to encourage and facilitate the development of biochemical science. That their aim has been fully realized, with a degree of success probably exceeding their most sanguine hopes, is in great measure attributable to the genius and the thoroughness of the early contributors, who set standards which unquestionably influenced the quality of biochemical research not only in North America but the world over.

It is interesting to view in retrospect some of the prominences on the horizon. The first paper in Volume 1 was an account of a chemical study of epinephrine by John J. Abel, who later attained renown by the crystallization of insulin. The first ten volumes include contributions from many of the other pioneer investigators whose work led to the establishment of biochemistry as an independent branch of science. Among these

are Francis G. Benedict, who with T. B. Osborne recorded the heats of combustion of vegetable proteins; Stanley R. Benedict, who initiated his long series of studies of analytical procedures for the estimation of glucose and other metabolites; Walter R. Bloor, who presented the first of his many studies of lipides; H. D. Dakin, whose work on biological oxidations profoundly influenced current views of the chemistry of metabolism; Otto Folin, whose name is associated with many of the analytical procedures employed in diagnosis; L. J. Henderson, who was one of the first to apply to biological problems the concept of hydrogen ion concentration and later stimulated accurate thinking by his scholarly articles on blood as a physicochemical system; Christian A. Herter, who reported studies on bacterial processes in the intestine; Treat B. Johnson, whose lifelong interest in the chemistry of pyrimidines elicited much information of biochemical significance; Walter Jones, whose investigations provided much of the present day knowledge of nucleic acids and nucleases; Phoebus A. Levene, who through many years worked indefatigably on the organic chemistry of a wide variety of compounds of biochemical interest; Jacques Loeb, whose studies of the biophysical effects of ions revolutionized various physiological concepts; Lafayette B. Mendel, whose work provided the basis of all nutritional research; T. B. Osborne, whose interest in vegetable proteins led to a long and extraordinarily fruitful collaboration with Mendel; A. N. Richards, whose later masterly studies of the complex processes of kidney secretion form an unsurpassed model of perfection; William C. Rose, whose long series of fundamental studies of nitrogen metabolism ultimately led to an accurate definition of the amino acid requirements of the rat and of man, and to the discovery of threonine, a previously unrecognized amino acid; Donald D. Van Slyke, who has provided so many ingenious and valuable technical methods for the study of proteins and of chemical processes of blood, and has added one more amino acid to the list of protein constituents.

Space permits the mention of only a few of the subsequent contributions to which the Journal may point with pride. During the second decade of the century the development of biochemical science was strongly influenced by the work on carotenoids by Leroy S. Palmer, on essential nutritional factors by E. V. McCollum, on the antineuritic vitamin by R. R. Williams, on pancreatic diabetes by Murlin, and on scurvy by Hess and by Hart and Steenbock. During the same period W. M. Clark introduced the function of pH into American biochemical thought, Kendall isolated thyroxine, and Dakin elaborated and applied new techniques for the estimation of amino acids in proteins.

The third decade witnessed great activity in the fields of nutrition and hormones. Studies of experimental rickets by Hess and by Steenbock led

to the recognition of the chemical nature of the antirachitic vitamins and their production by irradiation of certain sterols; G. O. and M. M. Burr demonstrated the dietary indispensability of the highly unsaturated fatty acids; R. R. Williams isolated the antineuritic vitamin and C. G. King determined the nature, and soon established the constitution, of the antiscorbutic vitamin; Steenbock, Hart and Elvehjem established the need for copper as well as iron in the prevention of dietary anemia; Doisy and Allen described the function of the ovarian hormone, which was soon isolated in crystalline form by Doisy and his collaborators; Gallagher and Koch purified the testicular hormone and studied its physiological effects. In the same period Mueller discovered methionine; Sumner crystallized urease; Conant carried out enlightening physicochemical studies of hemoglobin; Jacobs embarked on his long series of investigations of the chemical constitution of alkaloids; Anderson initiated his equally long study of the lipides of the tubercle bacillus; the Coris started their important work on carbohydrate metabolism; Heidelberger began his fruitful investigations in the field of immunochemistry; Michaelis drew attention to the biological significance of oxidation-reduction systems; du Vigneaud published his first paper on the chemistry of insulin, a study which ultimately led to his magnificent contributions on the synthesis of peptides, on the chemistry of sulfur-containing amino acids, on transmethylation, on the constitution of biotin, on the isolation, constitution and synthesis of the active principles of the posterior pituitary.

By 1931 the general pattern of reporting the results of biochemical investigations in the Journal had established itself and has not fundamentally changed during the past quarter century. On the other hand, the science itself has undergone many striking developments, and it is tempting to refer to some of the highlights in more recent years. In 1935 Schoenheimer and Rittenberg first applied isotopes to the study of intermediary metabolism. In 1936 Stanley isolated a virus in crystalline form, thereby disproving the idea that viruses were living organisms. In 1940 Dubos and Hotchkiss isolated gramicidin and tyrocidine, the first antibiotics to be well defined, and established the presence in them of D-amino acids. In 1941 Link isolated and determined the constitution of the compound responsible for the sweet clover disease of cattle. In 1943 Moore and Stein developed their valuable chromatographic technique for the separation of amino acids, and Woolley enunciated the principle of antagonism in the production of deficiency syndromes by the administration of analogues of vitamins and other essential nutrilites. In 1944 Craig described his highly effective method for the separation of closely related compounds by counter-current extraction, and Beadle introduced the use of artificially produced mutants of microorganisms for the biological assay of

vitamins, essential amino acids and similar compounds. At about the same time Max Bergmann and Fruton developed the use of synthetic peptides as substrates for the determination of enzyme specificity.

It is not surprising that over the years the character of the articles published in the Journal has undergone change. The originally wide scope, which embraced the cognate sciences, has necessarily contracted as the volume of purely biochemical contributions has increased, and in order to secure the publication of a maximum amount of factual information within the restricted field increasing emphasis on compactness, as well as clarity, of presentation has become essential. In one respect only has the task of the editors gradually grown lighter: the parallel development of other scientific disciplines has stimulated the establishment of other specialized journals which now shoulder their part of the general load. However, this gain has been more than offset by the constantly growing volume of genuinely biochemical contributions. Unless the Journal can by some miracle be permitted unrestricted growth, ever increasing selectivity with respect to subject matter and scientific quality will have to be exercised.

HANS T. CLARKE

PARTITION CHROMATOGRAPHY OF OXYTOCIN AND VASOPRESSIN

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In the course of investigating the hormones of the posterior pituitary gland in this laboratory, the methods of counter-current distribution (1–3), zone electrophoresis (4, 5), and, more recently, ion exchange chromatography (6) have been employed. By these means it has been possible to isolate oxytocin from beef and hog posterior pituitary glands (1, 3, 7), arginine vasopressin from beef glands (2, 8), and lysine vasopressin from hog glands (9). Synthetic oxytocin has been purified by means of counter-current distribution (10, 11) and has been found to behave identically with natural oxytocin when studied by zone electrophoresis (10, 11) and ion exchange chromatography (10, 6).

In order to characterize these hormones further and to develop methods for their isolation in small quantities from sources of limited availability, such as human pituitary glands, their behavior has been studied on partition columns of the type devised by Martin and Porter (12), with diatomaceous earth as the supporting phase and with solvent systems previously used for the counter-current distribution of the posterior pituitary hormones (1–3). With starting material of varying degrees of purity, prepared from beef and hog glands, it has been found that this type of column can be successfully employed to purify and characterize as little as 500 to 1000 U. S. P. units of the hormones, oxytocin and vasopressin. The method has also been applied to a comparison of natural and synthetic arginine vasopressin (13), in which it was found that the synthetic pressor activity occupied the same position in the chromatogram as did the natural hormone.

Methods

Preparation of Columns—Hyflo Super-Cel (Johns-Manville) was employed as the supporting phase. Prior to use, it was thoroughly washed with 3 N HCl, followed by water to remove impurities which appeared to cause inactivation of the hormones on the columns. The washing was repeated three times.

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The procedure adopted after some experimentation was to grind up 6.0 gm. of the Hyflo Super-Cel with 4.0 ml. of 0.1 per cent acetic acid saturated with 2-butanol. The wet Super-Cel was then stirred with 200 ml. of 2-butanol saturated with 0.1 per cent acetic acid. After stirring for an hour, the Super-Cel was allowed to settle, and the solvent was decanted. No lower phase should be present in the decanted solvent. The remaining slurry of Super-Cel was poured into a column, care being taken that no air bubbles were trapped in the column. For some experiments 50 ml. burettes fitted with Teflon stop-cocks were used, but, for the majority of the chromatograms, it was found convenient to use a 0.9 × 40 cm. tube of the type employed by Stein and Moore for starch chromatography (14). The columns were packed under 15 cm. of Hg pressure, and it was found that settling to constant volume was aided by gently tapping the column with a small mallet. The final volume of a column prepared with 6.0 gm. of Super-Cel was usually 20 ml., having a retention volume of 14 to 15 ml. In the case of the tubes 0.9 cm. in diameter, the height of the settled column was 32 cm. The flow rate was adjusted to 1.5 ml. per hour by attaching an external water reservoir to reduce the operating pressure on the column.

The settled column was treated with 8-hydroxyquinoline (14) to remove traces of metals still present in the Super-Cel. A 25 mg. sample of hydroxyquinoline was placed on the column in 2 ml. of solvent and then developed with 20 to 30 ml. of solvent. A green band moved down the column, which slowly changed to black. All of the hydroxyquinoline left the column after the passage of 1 hold-up volume of solvent.

In experiments in which columns of greater capacity were employed, the amounts of supporting phase and stationary phase were increased to 24 gm. of Super-Cel and 16 ml. of 0.1 per cent acetic acid saturated with 2-butanol, respectively. Tubes 1.9 cm. in diameter were used for these larger columns, and the column height after packing was 30 cm. When larger columns than this were employed it was difficult to keep the solvent and the column in equilibrium unless the columns were jacketed or kept in a constant temperature room. Small temperature variations of 2° or 3° did not cause difficulty with the columns having diameters of 0.9 cm. and 1.9 cm.

The material to be chromatographed was then placed on the column in 0.5 ml. of solvent in the case of the columns 0.9 cm. in diameter and washed into the Super-Cel with 3 portions of 0.2 ml. The reservoir was then filled with 150 ml. of solvent and the column mounted on the fraction collector. Fractions of 0.5 ml. were collected from columns of 0.9 cm. diameter, and the experiment was completed in about 3 days. In the case of the 1.9 cm. columns, 3 ml. fractions were collected.

After passage of 100 ml. of solvent through a column 0.9 cm. in diameter, by which time material having a partition coefficient less than 0.05 was coming off in the effluent, the solvent was changed to achieve complete recovery. For this purpose the *n*-butyl alcohol, *n*-propyl alcohol, 0.1 N HCl (1:2:1) solvent of Stein and Moore (15) was usually employed, or material remaining on the column was eluted with 0.1 per cent acetic acid.

Distribution of peptide material in the effluent fractions was determined with the modified Folin phenol reagent according to the method of Lowry, Rosebrough, Farr, and Randall (16). When the effluent fractions were 3 ml. as in the experiments with columns 1.9 cm. in diameter, the distribution of ultraviolet-absorbing material was determined at 275 mμ. Distribution of oxytocin activity was assayed by the chicken blood pressure method of Coon (17), while pressor assays were carried out in anesthetized rats injected with Dibenamine (18).

Results

Experiments with Partially Purified Preparations—A partially purified beef preparation, having a potency of 60 pressor units per mg., was chromatographed on a 1.9 × 30 cm. column. 43 mg. of material were dissolved in 1 ml. of solvent and placed on the column, which was then developed with 400 ml. of solvent. The flow rate was adjusted to 4 ml. per hour, and the experiment was completed in 2 days. As shown in Fig. 1, the pressor activity emerged from the column at 200 effluent ml., with the peak of activity at 320 ml. The K value[1] was estimated to be 0.1 for this peak, which is the same as that found for beef vasopressin when the same starting material was distributed in the Craig counter-current apparatus with the same solvent system.[2] The specific activity in the peak fraction was estimated to be about 125 to 150 units per mg. in solution. This represents a 2-fold purification. To achieve the same degree of purification by counter-current distribution requires the application of at least 1000 transfers.[2] Approximately 50 per cent of the activity placed on the column was recovered in the peak having a K of 0.1.

In another experiment a preparation of beef vasopressin which had been partially purified by preparative electrophoresis was chromatographed on a 0.9 × 30 cm. column. 3.66 mg. of this preparation, which had a potency of 280 units per mg., were developed with the 2-butanol-0.1 per cent acetic acid solvent. The effluent curves of Fig. 2 indicated some impurities at effluent volumes of 20 and 43 ml., respectively, as well as a trailing shoulder

[1] Partition coefficients were calculated from the formula $K = V_s/(V_e - V_A)$, where V_s is the volume of stationary phase, V_e the effluent volume, and V_A the retention volume of the column (19).

[2] P. Condliffe and V. du Vigneaud, unpublished data.

which had a lower specific activity than the peak tubes. Recoveries of pressor activity were 70 per cent in the experiment shown and 75 per cent in another chromatogram of the same preparation. When the effluent fractions between 50 and 75 ml. were pooled and lyophilized, the dry weight

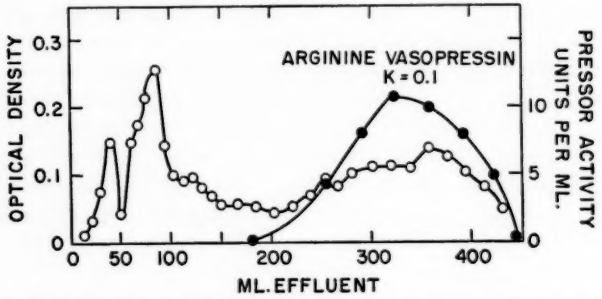

FIG. 1. Chromatogram of partially purified beef vasopressin. ○, ultraviolet absorption at 275 mμ; ●, vasopressin activity. 43 mg. of combined Kamm "e" and "f" fractions, assaying 60 pressor units per mg., were placed on a column 1.8 × 30 cm.

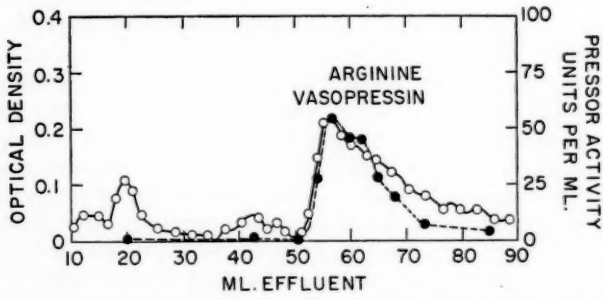

FIG. 2. Chromatogram of partially purified beef vasopressin. ○, color with the reagent of Lowry *et al.*; ●, vasopressin activity. 3.66 mg. of a fraction partially purified by electrophoresis, which assayed 280 pressor units per mg., were placed on a column 0.9 × 30 cm.

recovered was 2 mg. The activity recovered in these same fractions was 680 units in solution, giving a specific activity of 340 units per mg. Quantitative amino acid analysis of the lyophilized material gave the same amino acids previously found for beef vasopressin (2) in the molar ratio of 1:1 to each other. No leucine or isoleucine was detected in the recovered fractions, although the starting material had contained 0.15 mole of each to every mole of the eight amino acids usually found in beef vasopressin, as

well as traces of serine, methionine, valine, and histidine. Thus, additional indication of purification was afforded.

Results similar to these were found with other partially purified vasopressin preparations of beef origin. The specific activity of the preparations studied ranged from 60 to 280 units per mg. It was found that with starting material of 100 units per mg., or better, it was possible to recover fractions having a potency of 300 to 340 units per mg. With as little as 3 mg., a sufficient amount of the highly potent fractions could be recovered to determine the amino acid content either by paper chromatography or by quantitative starch column chromatography.

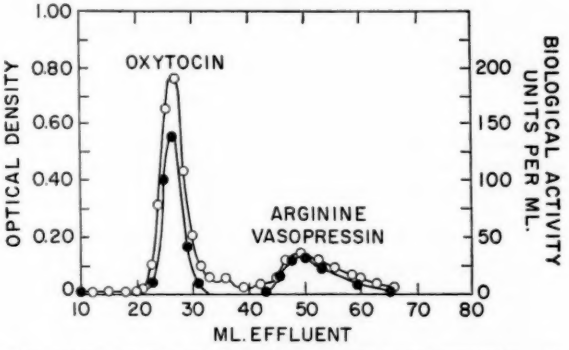

FIG. 3. Chromatogram of a mixture of vasopressin and oxytocin. ○, color with the reagent of Lowry *et al.*; ●, vasopressin activity; ●, oxytocic activity. 2.2 mg. of arginine beef vasopressin, assaying 400 units per mg., with 1.81 mg. of beef oxytocin, assaying 500 units per mg. Column, 0.9 × 30 cm.

Experiments with Purified Hormones—Experiments with partially purified preparations of vasopressin showed that arginine vasopressin had a partition coefficient of 0.1 in the solvent system 2-butanol-0.1 per cent acetic acid. A partition coefficient of 0.4 has been found for oxytocin when it is subjected to counter-current distribution in this solvent system. Accordingly, the resolving power of the column was tested by chromatographing a mixture of highly purified vasopressin and oxytocin having potencies of 400 and 500 units per mg., respectively. 2.2 mg. of arginine vasopressin were mixed with 1.81 mg. of oxytocin of beef origin. The mixed powders were dissolved in 1 ml. solvent and placed on a column 0.9 × 30 cm. The flow rate was adjusted to 1.5 ml. per hour, and 0.5 ml. fractions were collected. The entire experiment was completed within 48 hours. The results presented in Fig. 3 indicate that oxytocin and arginine vasopressin can be completely resolved on this column. Oxytocin appeared in the

expected position ahead of vasopressin, and virtually complete recovery of both hormones was achieved, 100 per cent for oxytocic activity and 85 per cent for pressor activity. The potency was unchanged in either case.

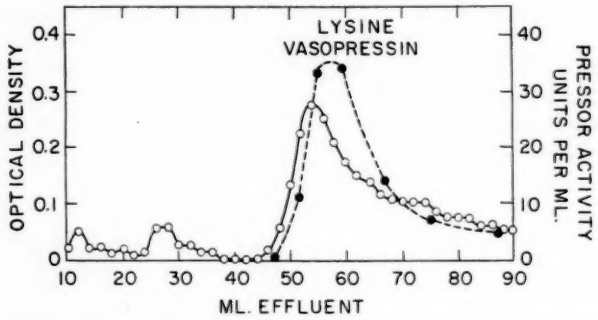

FIG. 4. Chromatogram of lysine vasopressin. ○, color with the reagent of Lowry *et al.*; ●, vasopressin activity. 2.2 mg. of lysine vasopressin which assayed 275 to 295 units per mg. on a column 0.9 × 30 cm.

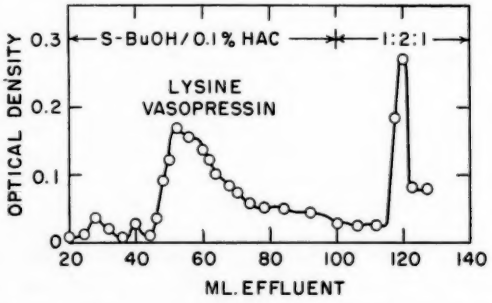

FIG. 5. Chromatogram of lysine vasopressin. ○, color with the reagent of Lowry *et al.*; 2.79 mg. of lysine vasopressin, which assayed 275 to 295 units per mg. on a column 0.9 × 30 cm. 1:2:1 refers to the second solvent mixture, as specified in the text.

The behavior of lysine vasopressin was then studied in order to learn whether the column was capable of separating it from arginine vasopressin. It appeared from counter-current distribution studies that the partition coefficient differed slightly from that of arginine vasopressin.[3] Accord-

[3] The author is greatly indebted to Dr. Darrell N. Ward, who prepared the lysine vasopressin employed in this work. These preparations are the most potent obtained to date. The details of purification will be published in a separate communication from this laboratory by D. N. Ward and V. du Vigneaud.

ingly, 2.2 mg. of a purified lysine vasopressin preparation were placed on a column 0.9 × 30 cm. The specific activity of the preparation was about 275 to 295 units per mg. The effluent curves for the color reaction and pressor activity indicated that the hormone had a K value of 0.09, which is very close to that found for arginine vasopressin. However, the recovery of activity was appreciably lower than found for the other hormone, less than 50 per cent of the total amount of material placed on the column. In other experiments the K value varied from 0.1 to 0.07, and there was always a considerable amount of trailing after the main peak had

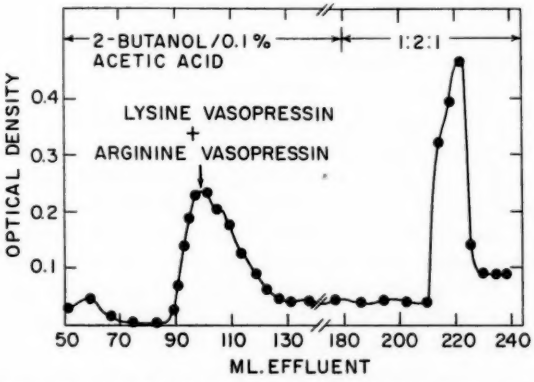

FIG. 6. Chromatogram of a mixture of lysine and arginine vasopressin. ●, color with the reagent of Lowry *et al.*; 3.64 mg. of arginine vasopressin, assaying 400 units per mg., mixed with 3.45 mg. of lysine vasopressin, assaying 275 units per mg. Column, 0.9 × 45 cm. 1:2:1 refers to the second solvent mixture, as specified in the text.

emerged from the column, as shown in Fig. 4. It seemed probable that part of the hormone was being bound on the column. It was possible to recover the missing activity by washing the column with 0.1 per cent acetic acid. The sharpest elution peak was obtained by using 1:2:1 1-butanol-1-propanol-0.1 N HCl as the second solvent. By this means close to 90 per cent recovery of activity was achieved, although only 40 per cent appeared in the first peak, with a K of 0.1. A typical experiment is shown in Fig. 5.

Experiments with mixtures of lysine and arginine vasopressin and with lysine vasopressin and oxytocin were also carried out. While it was possible to separate lysine vasopressin and oxytocin, the experiments with the two vasopressins were not uniformly successful, owing to the closeness of their K values, although some degree of separation was obtained in a few

experiments. It was again found in these experiments that, while arginine vasopressin trailed only slightly on the column, 80 to 90 per cent recovery being obtained with the first solvent, a large proportion of the lysine vasopressin failed to emerge at the expected position and was recovered by elution with a strongly acidic solvent. In a typical experiment illustrated in Fig. 6, it was found that the first peak to emerge at 100 effluent ml. contained both lysine and arginine when a portion of the recovered material was hydrolyzed and the hydrolysate examined by paper chromatography. The second peak, at 42 ml. after the solvent change, was recovered, and the amino acids were determined by starch chromatography. The ratio of lysine to arginine in the hydrolysate of this peak was 4:1. In other experiments paper chromatography of hydrolysates of the second peak failed to reveal any but traces of arginine.

DISCUSSION

Previous work in this laboratory has shown that the hormones of the posterior pituitary can be highly purified by counter-current distribution (1–3). In this paper it has been demonstrated that these hormones can be partitioned on columns with Hyflo Super-Cel as the supporting phase and a solvent system similar to that previously used for counter-current distribution. Although material of as high a degree of potency as obtained by other methods has not yet been isolated, the method offers considerable promise.

In the case of counter-current distribution, the smallest amount of activity which can be conveniently distributed is between 20,000 and 40,000 units of oxytocic or pressor activity, representing 40 to 80 mg. quantities of the purified hormones. To obtain this much activity from natural sources requires pituitary glands from several thousand animals.

By means of this small partition column, as little as 500 to 1000 units (1 to 2 mg.) of oxytocin or vasopressin may be chromatographed, and the amino acid composition and biological potency of the hormone peaks can be estimated. This amount of activity represents the amount which can be recovered from 50 to 100 beef glands or 200 to 400 hog glands.

Human posterior pituitary glands are of comparable potency[2] to hog and beef glands and are of intermediate size. To accumulate several thousand human glands would be a difficult undertaking, but it is possible in the course of time to accumulate the few hundred necessary for chromatography on a column of the type described in this paper.

The choice of solvents was dictated by several considerations. First, the partition coefficients of oxytocin and vasopressin had to lie within the range 0.05 to 0.5 for successful chromatography. Second, these K values had to differ sufficiently to separate the oxytocic and pressor activities.

Third, the solvent used should, if possible, be volatile. As already mentioned, it was found by Livermore and du Vigneaud (1) that oxytocin had a K of 0.4 in 2-butanol-dilute acetic acid, while that of vasopressin had been found in this laboratory to be approximately 0.1. This solvent system is a simple one in which the hormones are stable. Other systems were considered, such as 2-butanol-*p*-toluenesulfonic acid of various proportions. Some experiments were carried out with such systems, but, in order to carry out amino acid analyses on the effluent fractions, it was necessary to remove the acid on an ion exchange column, which frequently led to loss of hormonal activity. Thus, it seemed that the solvent system chosen would be the most satisfactory for the purpose at hand, and this was found to be the case.

SUMMARY

A chromatographic procedure was developed for the isolation of 500 to 1000 units of oxytocin and vasopressin. This amount of activity, equivalent to 1 to 2 mg. of the highly purified hormones, was found to be sufficient for the estimation of biological potency and amino acid content of the active fractions. The procedure was applied to the fractionation of partially purified posterior pituitary preparations of beef origin and to mixtures of the purified hormones.

The author wishes to thank Professor du Vigneaud for his generous support, encouragement, and wise counsel while this work was being carried out. The author also acknowledges the able assistance of Miss Joan Krakow and Miss Sylvia Kirsimagi in the performance of the bioassays and wishes to thank Mr. Richard J. Murphy for his assistance with the starch column analyses. Appreciation is expressed to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this study.

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CHROMATOGRAPHIC IDENTIFICATION OF BOUND GLUTAMIC ACID IN THE PHOSPHATIDE FRACTION FROM LIVER*

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The phosphatides (1) are a group of substances having in common the presence of fatty acids and esters of phosphoric acid among their constituents. This composition confers upon them solubility properties closely akin to those of the simple lipides. In the phosphatide fraction, isolated upon the basis of characteristic solubility properties, we have identified glutamic acid as a component liberated by acid hydrolysis. This investigation extends the information on uncharacterized phosphatide constituents reported in a previous study of the metabolism of ethanolamine-1,2-C^{14} (2).

EXPERIMENTAL

Sources of Lipides—The lipides were extracted from the livers of male rats of the Long-Evans strain which were sacrificed 4 hours after receiving an intraperitoneal injection of 3 μc. of ethanolamine-1,2-C^{14} (2), or from 500 mg. of liver slices which were incubated aerobically for 2 hours at 37° in the presence of 5 ml. of Krebs-Ringer-phosphate solution at pH 7.4 and substrate concentrations of 3.8×10^{-4} M ethanolamine-1,2-C^{14} and 3×10^{-4} M methionine. The incubated tissue was denatured by the addition of 2 ml. of 3:1 ethyl alcohol-ethyl ether; the entire mixture was then raised to a temperature of 75° while being agitated.

Phospholipide Extraction Procedure—Phospholipides were prepared as described below by a modification of methods reported previously from this laboratory (2) and those of Fraenkel and Neubauer (3) and Parnas (4).

The liver excised from the intact animal, or the liver slices plus the incubation medium and denaturant, were transferred to a homogenizing tube and homogenized in the presence of a mixture of 3:1 ethyl alcohol-

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ethyl ether. In the case of experiments *in vivo* the homogenization was performed in the presence of pure ethyl alcohol-ethyl ether, whereas in experiments *in vitro* the homogenizing medium was diluted with the incubation medium. The homogenate was then transferred to heavy walled Pyrex centrifuge tubes and centrifuged for 5 minutes at 1100 × *g*, following which the supernatant fluid was transferred to 200 ml. Erlenmeyer flasks.

The initial homogenization was followed by three more complete extractions which consisted of the treatment of approximately 500 mg. of homogenized liver tissue by 10 ml. of ethyl alcohol-ethyl ether at 65° for 10 minutes. The extraction was performed in the centrifuge tube, in which a fine stirring rod was inserted to prevent bumping. After each extraction the material was centrifuged and the supernatant solution was transferred to the extract resulting from the initial homogenization. A fourth and final extraction was made with ethyl ether. The combined extracts were evaporated *in vacuo* to complete dryness.

The dry residue was then extracted with 10 ml. of petroleum ether three times; the extracts were centrifuged and the supernatant fluid was carefully transferred to another centrifuge tube without carrying over any visible sediment. This extract was evaporated to about 0.5 ml.

All procedures for phospholipide precipitation were carried out in a cold room at 3°. The phospholipides were precipitated by the addition of 10 ml. of acetone. The sample was allowed to stand for half an hour and was then centrifuged. The supernatant fluid was decanted and the phospholipide sediment was allowed to drain by inverting the centrifuge tubes and allowing them to stand in this position for several minutes. The phospholipides were redissolved by the addition of 1 ml. of petroleum ether. The petroleum ether was evaporated to 0.5 ml. and the entire procedure of precipitation, drainage, and redissolving repeated three to five times.

For hydrolysis of the phospholipides, the precipitate was dissolved in petroleum ether and transferred to a digestion flask. The petroleum ether was evaporated, and the residue was hydrolyzed for 9 hours in 35 ml. of boiling 3.6 per cent HCl. Following hydrolysis, the hydrolysate was filtered and evaporated to dryness *in vacuo*.

Identification of Glutamic Acid by Column Chromatography—The dried hydrolysate was dissolved in a minimal volume of distilled water, transferred to a 9 mm. × 120 cm. column of the cation exchange resin, Dowex 50 (250 to 500 mesh), and eluted with 1.5 N HCl at the rate of 0.1 ml. per minute (see Fig. 4 (2)). The fractions, corresponding to about 1.5 ml., were collected and dried in polyethylene planchets, and from the data effluent curves were plotted in terms of radioactivity and ninhydrin color (2). In all instances a ninhydrin-positive compound was present, emerging

just after serine. Tests under standard conditions gave glutamic acid at Fractions 56 to 66; the peak of the curve of elution occurred at Fraction 60 or 61. Serine emerged at Fractions 46 to 56, with the peak at Fraction 51. When unknown hydrolysates were run, two ninhydrin-positive compounds were always eluted at the positions corresponding to serine and glutamic acid.

Identification by Paper Chromatography—Unidimensional paper chromatographs of the hydrolysate were run for 24 to 48 hours on Whatman No. 4 paper. Saturated aqueous phenol and *n*-butanol-acetic acid-water (100:21:50) were used as solvents. Color was developed at 80° with 0.1 per cent ninhydrin and 2 per cent collidine in 95 per cent ethyl alcohol. In all instances glutamic acid and serine standards were chromatographed in parallel with the hydrolysate. Analysis of the distribution of radioactivity on paper chromatographs of the hydrolysate with a scanning gas flow counter demonstrated no measurable radioactivity in the glutamic acid fraction. The migration of standard glutamic acid coincided with the migration of the unknown compound for each solvent under varying degrees of temperature and variation in the duration of the chromatographic run. The R_F value of the unknown compound (and of glutamic acid) was 0.31 in phenol and 0.37 in butanol-acetic acid. The same pattern, but of a heavier color intensity, was obtained with hydrolysates of material from petroleum ether extracts not submitted to the acetone precipitation step.

In the course of these studies the pattern of migration of D-glutamic acid was observed to be characteristically different from that of L-glutamic acid. Upon development of the color with ninhydrin, the D-glutamic acid spot gave a symmetrical pattern, whereas L-glutamic acid yielded an asymmetric pattern characterized by a U-shaped ninhydrin-negative area inserted at the top of an otherwise symmetrical ninhydrin-positive area. The samples chromatographed were all obtained from one commercial supply house. The pattern of asymmetry was noted usually upon chromatography with aqueous phenol in a range of higher concentrations of L-glutamic acid. It was never noted with D-glutamic acid in the higher concentration ranges. This observation suggests that the unknown compound is L-glutamic acid, since it migrated with formation of the same pattern when greater concentrations were chromatographed.

Control Experiments on Glutamic Acid, Glutamine, and Pyrrolidonecarboxylic Acid As Possible Contaminants—In view of the possibility of the glutamic acid accompanying the other phosphatide constituents as a contaminant, several tests were carried out on the solubility characteristics of crystalline glutamic acid in the system employed for phospholipide extraction and precipitation.

Crystalline DL-glutamic acid was found to be sufficiently soluble in 3:1 ethyl alcohol-ethyl ether to give the color observed in the ninhydrin test. In this procedure 100 mg. of DL-glutamic acid were thoroughly mixed with 10 ml. of a 3:1 ethyl alcohol-ethyl ether mixture. This preparation was then centrifuged and the supernatant fluid tested for the presence of glutamic acid by chromatography on paper and by developing with ninhydrin. When the same test was repeated with a petroleum ether extract, a reaction with ninhydrin could not be obtained. In addition, when the 10 ml. of petroleum ether were carefully decanted, evaporated to dryness, and the residue resulting from the dried petroleum ether extract taken up in a small volume of water and applied to chromatographic paper, a negative ninhydrin test was obtained, thus indicating that the solubility in 10 ml. of petroleum ether was not sufficient to produce a ninhydrin reaction.

Since free glutamic acid could have been converted to pyrrolidonecarboxylic acid by the denaturation procedure for the incubation *in vitro*, or since pyrrolidonecarboxylic acid may possibly be a constituent of tissues, it was necessary to determine whether pyrrolidonecarboxylic acid was carried along in the petroleum ether extraction procedure. Glutamine was also considered in view of the report (5) that it may be carried through in a petroleum ether extraction procedure.

Pyrrolidonecarboxylic acid was prepared by refluxing 340 mg. of L-glutamic acid for 10 hours in 12 ml. of distilled water. The refluxed material which contained pyrrolidonecarboxylic acid and glutamic acid was vacuum-dried at 65° for 15 hours.

Pyrrolidonecarboxylic acid was identified in the synthetic preparation by chromatography on Whatman No. 1 paper with a solvent composed of 12 gm. of phenol in 4 ml. of H_2O plus 1 per cent by weight of formic acid (88 per cent). The pyrrolidonecarboxylic acid appears as a yellow spot against a blue-green background upon spraying with slightly alkaline (pH 7.3) bromocresol green in 95 per cent ethyl alcohol (6). Glutamine could be identified with difficulty by the dye, but was very easily identified by spraying the dye-developed paper with ninhydrin.

The dried pyrrolidonecarboxylic acid preparation, with added glutamine, was extracted with petroleum ether and the extract chromatographed. The results in all cases were completely negative.

Consideration was given to the possibility that the presence of lipide material rendered glutamine and pyrrolidonecarboxylic acid soluble. Consequently the complete phospholipide extraction procedure was carried out, beginning with homogenization of rat liver slices in the presence of pyrrolidonecarboxylic acid, glutamine, and saline solution. As before, when the petroleum ether extract was chromatographed on paper, the results were all negative. Collectively these observations may be taken to

indicate that the substance chromatographically identified as glutamic acid was in bound form in the lipide extract.

DISCUSSION

An analysis of the evidence presented indicates the existence of a glutamic acid complex which is lipide in nature. If glutamic acid is a *real* component of the phospholipide molecule, we are then confronted with the presence of a new type of phospholipide. It is possible, but not highly probable, that some ninhydrin-positive component of lipides or phospholipides, not identifiable with glutamic acid, possesses the same rate of migration on a Dowex 50 column or the same R_F value with aqueous phenol or butanol-acetic acid-water solvent as does glutamic acid. If more definitive analyses demonstrate that this compound is not glutamic acid, it would still, at this time, appear valid to assume some new component of phospholipides which bears a structural similarity to glutamic acid. The presence of glutamic acid in a phospholipide could be explained within the confines of known phosphatidic structures if one considers glutamic acid as being present in the lipide as a phosphatidyl peptide; *e.g.*, a peptide of serine and glutamic acid. This view-point appears warranted from the observations of Wynn and Williams on blood plasma (5) and the studies of Folch on neurokeratin (7).

SUMMARY

Column and paper chromatographic evidence is presented to indicate the occurrence of glutamic acid as a component of the phosphatides of rat liver.

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EFFECT OF ANDROGENS AND GROWTH AND OTHER HORMONES ON ETHIONINE FATTY LIVER IN RATS*

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Ethionine (γ-ethylthio-α-aminobutyric acid), an analogue of methionine, induces fatty livers in fasting female (1, 2) and castrated male (3) rats but not in intact males and females pretreated with testosterone (3). A relationship between androgens and the response of rats to ethionine was thus suggested.

The present study was undertaken in an attempt to confirm and extend the foregoing observations and to determine what effect other hormones might have on the development of the fatty liver. Such information might throw light on the pathogenesis of the hepatic change and might also give clues to the mechanism of action of some hormones. It was found that pretreatment with androgens or growth hormone depressed the ethionine-induced rise in liver lipide; cortisone and corticotropin (ACTH) slightly enhanced it. The degree of protection varied with dosage.

EXPERIMENTAL

Adult female Fisher rats, 140 to 160 gm., line-bred in this laboratory, were ovariectomized and, beginning 1 week later, injected daily with the hormone preparations for 3 weeks unless otherwise indicated. On the 20th day, food was removed and 1 mg. of DL-ethionine per gm. of body weight was injected intraperitoneally in aqueous solution (25 mg. per ml.) in three divided doses, 2½ hours apart. Generally, each preparation was administered to ten rats in two groups of five. The animals were killed with ether between 24 and 26 hours after the first injection of ethionine. The liver was rapidly removed and weighed, and an aliquot (about 3.5 gm.) for lipide analysis was immediately frozen. The liver aliquot was thawed,

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† Scholar in Cancer Research of the American Cancer Society as recommended by the Committee on Growth of the National Research Council.

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ground with anhydrous Na_2SO_4, and extracted with chloroform for 24 hours, as previously described (1). The petroleum ether-soluble material from this chloroform extract was weighed and is referred to as total lipide. In almost every instance, the chloroform-soluble material was completely soluble in petroleum ether.

Hormone Preparations—Testosterone propionate was used as a solution in sesame oil. The other steroids used, named according to the nomenclature proposed by Fieser and Fieser (4), and with the number in parentheses representing the designation given by Selye (5), are 17α-methyltestosterone (244) and Δ^5-androstene-3β,17β-diol (192), each in an emulsion, estradiol-17β (34) and 11-deoxycorticosterone acetate (354) in sesame oil, and cortisone acetate (378) as a suspension in saline. Sodium L-thyroxine was given in propylene glycol solution. The crystalline zinc insulin, prolactin, thyroid-stimulating hormone (TSH), and the various growth hormones were given in saline. ACTH was in the form of the standard gel preparation.

Results

Androgens—Rats pretreated with testosterone propionate in daily doses of 2.5 mg. or more showed less liver lipide following ethionine administration than control animals (Table I), the larger doses being associated with progressive decrease in liver lipide. Androstenediol, although a weaker androgen, was approximately as effective as equal doses of testosterone propionate in protecting against the rise in liver lipide *concentration*, but less effective in preventing the rise in *total* lipides per liver (Table I). The discrepancy is due to an increase in liver weight proportional to the dose of androstenediol. Methyltestosterone had an effect similar to that of the other androgens (Table I).

Growth Hormone—Pituitary growth hormone protected the liver against the fatty change (Table I). On the whole, this hormone was more effective than androgens given in the same dosages, but there was less consistency. In some experiments, both the beef and the hog growth hormone preparations showed greater protective effects than the most active androgen. In other experiments, the same preparations in the same doses were less effective.

The beef hormone was superior to the hog preparation in protecting against ethionine. Although both are active growth hormones, each is contaminated with a different hormone. For example, the hog hormone, Armour No. R285174, contains a significant amount of ACTH, whereas the beef preparation, Armour No. R285110, contains thyroid-stimulating hormone. Since ACTH increases the level of the liver lipides in ethionine-treated rats (Table I), it probably tends to counteract the effect of the

growth hormone in the hog preparation. On the other hand, thyroid-stimulating hormone appears not to have any effect upon the ethionine fatty liver (Table I), thus allowing the full effect of the beef preparation.

TABLE I

Effect of Various Hormones on Liver in Ethionine-Treated and Control Rats

Pretreatment	Dose	Ethionine*	No. of rats	Total liver lipide		Liver weight
	mg. per day			*gm. per 100 gm. wet liver*	*mg. per liver per 100 gm. body weight*	*gm. per 100 gm. body weight*
None		−	14†	5.8 ± 0.4‡	185 ± 8‡	3.66 ± 0.17‡
		+	41†	13.6§ ± 0.5	498§ ± 14	3.79 ± 0.07
Androgens						
Testosterone propionate	15	−	10	4.0§ ± 0.2	188¶ ± 5	4.68§ ± 0.14
	5	−	5	4.4‖ ± 0.5	144‖ ± 14	3.38¶ ± 0.08
	15	+	9	6.0§ ± 0.4	251§ ± 11	4.22‖ ± 0.15
	10	+	10	6.6§ ± 0.4	266§ ± 13	4.07¶ ± 0.15
	5	+	9	7.5§ ± 0.7	282§ ± 24	3.76¶ ± 0.12
	2.5	+	3	9.3§ ± 0.5	373‖ ± 26	3.99¶ ± 0.09
	0.25	+	5	11.9‖ ± 0.5	428¶ ± 29	3.91¶ ± 0.10
	0.10	+	3	12.4¶ ± 0.8	450¶ ± 27	3.92¶ ± 0.07
	0.025	+	4	15.0¶ ± 1.2	564¶ ± 48	3.87¶ ± 0.12
Androstenediol	10	−	5	5.6¶ ± 0.3	161¶ ± 19	
	15	+	10	5.5§ ± 0.3	309§ ± 12	6.19§ ± 0.21
	10	+	9	5.8§ ± 0.4	300§ ± 8	5.71§ ± 0.15
	5	+	8	6.9§ ± 0.7	303§ ± 37	4.74§ ± 0.07
	2.5	+	9	7.2§ ± 0.4	309§ ± 10	4.67§ ± 0.14
	1.0	+	7	13.6¶ ± 0.4	463¶ ± 15	3.41¶ ± 0.10
	0.25	+	8	15.5‖ ± 0.8	474¶ ± 23	3.08§ ± 0.10
	0.10	+	8	13.6¶ ± 0.3	448¶ ± 20	3.31§ ± 0.14
Methyltestosterone	5	−	10	3.8§ ± 0.3	121§ ± 7	3.27¶ ± 0.16
	5	+	8	6.6§ ± 0.7	265§ ± 26	4.05‖ ± 0.09
	1	+	10	10.3¶ ± 1.1	404‖ ± 44	3.99¶ ± 0.07
Growth hormone						
Hog growth hormone R285174	1.0	−	10	5.2¶ ± 0.2	149§ ± 4	2.90§ ± 0.10
	1.0	+	18	9.1§ ± 0.8	343§ ± 25	3.97¶ ± 0.09
Beef growth hormone R285110	2.5	−	5	3.8§ ± 0.1	143§ ± 4	3.74¶ ± 0.09
	1.0	−	15	4.0§ ± 0.1	128§ ± 3	3.21¶ ± 0.21
	2.5	+	10	4.6§ ± 0.6	286§ ± 16	3.83¶ ± 0.13
	1.0	+	10	6.9§ ± 0.5	245§ ± 11	3.57‖ ± 0.06
	0.5	+	9	10.4‖ ± 1.1	369§ ± 36	3.87¶ ± 0.13
	0.25	+	9	10.4‖ ± 1.3	370‖ ± 46	3.58¶ ± 0.07

TABLE I—*Concluded*

Pretreatment	Dose	Ethionine*	No. of rats	Total liver lipide		Liver weight
Other hormones						
	mg. per day			*gm. per 100 gm. wet liver*	*mg. per liver per 100 gm. body weight*	*gm. per 100 gm. body weight*
Estradiol-17β	50 γ	−	10	4.1§ ± 0.1	171¶ ± 5	4.19‖ ± 0.15
	50 "	+	20	12.1¶ ± 0.8	589§ ± 14	4.90§ ± 0.10
DOCA**	0.5 mg.	+	4	12.6¶ ± 1.0	432¶ ± 33	3.67¶ ± 0.11
Cortisone	1.25 mg.	−	9	5.2¶ ± 0.5	168¶ ± 13	3.27¶ ± 0.09
	1.25 "	+	16	16.4§ ± 0.5	600§ ± 23	3.65¶ ± 0.09
ACTH R491205	5 Armour units	−	10	4.2§ ± 0.3	135§ ± 8	3.16§ ± 0.08
	5 " "	+	7	15.9§ ± 0.4	578‖ ± 16	3.64¶ ± 0.10
TSH BT-317-147	1 mg.	−	10	4.6§ ± 0.2	146§ ± 6	3.21‖ ± 0.08
	1 "	+	9	13.3¶ ± 0.9	542¶ ± 35	4.09§ ± 0.07
L-Thyroxine	100 γ	−	10	3.2§ ± 0.2	137§ ± 9	3.85¶ ± 0.11
	100 "	+	8	9.4§ ± 0.7	399‖ ± 33	4.25§ ± 0.13
Prolactin BP-369-149	2.5 mg.	+	8	15.2¶ ± 0.8	591‖ ± 35	3.89¶ ± 0.10
Zn insulin	10 units	−	6	4.9‖ ± 0.2	155‖ ± 6	2.16§ ± 0.06
	10 "	+	4	15.1¶ ± 0.7	559‖ ± 20	3.64¶ ± 0.05

* 1 mg. of DL-ethionine per gm. of body weight (see the text).

† Includes animals receiving saline, sesame oil, and other suspending and emulsifying agents. No differences were noted between groups of rats receiving these different media.

‡ Standard error of the mean.

§ $P < 0.01$ (highly significant).

¶ $P > 0.05$ (not significant).

‖ $P < 0.05$ (significant).

** 11-Deoxycorticosterone acetate.

Thus these contaminants probably account for the general superiority of the beef hormone over the hog hormone in protecting against the fatty liver.

The results with androgens and growth hormone in rats not receiving ethionine are noteworthy. With testosterone propionate, methyltestosterone, and the beef growth hormone, there was a significant decrease in liver lipide concentration (Table I). This is due in one testosterone group (15 mg.) to a large increase in liver weight. However, in the other groups, this effect appears to be related to a more direct influence upon liver lipide, since no increase in liver weight occurred.

Duration of Pretreatment—A 3 day period of pretreatment with testosterone propionate was ineffective in decreasing the ethionine-induced fatty liver, whereas maximal effect was obtained after 7 days (Table II). Administration of beef growth hormone for 7 or 14 days afforded less protection than a 21 day period of pretreatment (Table II).

Other Hormones—The results in Table I show that estradiol-17β, 11-deoxycorticosterone acetate, TSH, prolactin, or zinc insulin in the doses used had no apparent effect upon the fatty liver induced by ethionine. The lack of effect of estradiol on the liver lipide concentration, despite the large increase in liver weight, is noteworthy. It is interesting that control animals receiving only TSH showed no rise in liver lipide, even though this preparation is reported to be active in increasing liver lipides in fasting mice.[1] Cortisone acetate and ACTH produced a significant increase in liver lipide in ethionine-treated animals. In control rats, ACTH decreased the liver lipide as well as the liver weight.

The results with sodium L-thyroxine are difficult to interpret. The liver lipide values for animals treated with ethionine and thyroxine are considerably lower than those for animals receiving ethionine but no thyroxine.

TABLE II

Influence of Duration of Treatment with Testosterone Propionate and Growth Hormone upon Liver of Ethionine-Treated Rats

Duration of treatment	Hormone	No. of rats	Total liver lipide		Liver weight
days			*gm. per 100 gm. wet liver*	*mg. per liver per 100 gm. body weight*	*gm. per 100 gm. body weight*
21	Testosterone	10	6.6 ± 0.4†	266 ± 13†	4.07 ± 0.15†
14	propionate*	4	5.1 ± 0.2	212 ± 8	4.16 ± 0.07
7		5	6.2 ± 0.8	245 ± 26	3.95 ± 0.06
3		4	13.8 ± 1.7	533 ± 79	3.83 ± 0.07
21	Beef growth hor-	10	6.9 ± 0.5	245 ± 11	3.57 ± 0.06
14	mone	9	10.6 ± 0.6	381 ± 23	3.58 ± 0.07
7	R285110‡	10	9.1 ± 1.0	306 ± 31	3.42 ± 0.08
3		10	13.9 ± 0.9	498 ± 30	3.60 ± 0.12

* 10 mg. per day. All animals received ethionine (see the text).
† Standard error of the mean.
‡ 1 mg. per day. All animals received ethionine (see the text).

However, the value is about 3 times greater than those for the thyroxine-treated controls, which are the lowest of all control values. The discrepancy between the results with thyroxine and TSH in their effects on liver lipide in control and ethionine-treated rats could be due to the relatively greater dose of thyroxine used.

DISCUSSION

Three androgens, testosterone propionate, an androstenediol, and methyltestosterone, although differing in androgenic potency, were almost

[1] Steelman, S. L., personal communication.

equally effective in decreasing the liver lipide in ovariectomized female rats given ethionine. Increasing doses of the androgens produced decreasing liver lipide values. In most experiments, the protection was not complete, since the liver lipide levels were not as low as those in control animals given androgens alone.

The interesting large increase in liver weight produced by the androstenediol was proportionate to dosage and paralleled the protection against fatty liver. The increase in liver weight *per se* did not appear to be the important factor, since estradiol also produced a large increase in liver weight and yet exerted no protective action. Nor does it appear that the liver composition was grossly altered by the androstenediol, since normal percentages of water, total protein, and lipide were found.[2]

The results with hypophyseal growth hormone and thyroid-stimulating hormone are of special interest. Many previous studies have shown that growth hormone preparations increase the level of liver lipides in short term experiments on fasting mice (*cf.* (6)). Hypophyseal tissue is also reported to contain other fractions capable of "mobilizing fat" to the liver in mice (*cf.* (6)). Such fractions have been called adipokinin. The thyroid-stimulating hormone used in our experiments had potent adipokinin activity.[1] Yet, in the present study, neither growth hormone nor thyroid-stimulating hormone produced an elevation in liver lipides in control rats, and growth hormone significantly decreased the liver lipide level in ethionine-treated animals.

To prevent the ethionine fatty liver, both testosterone propionate and growth hormone must be administered over a definite period of time before ethionine is given. For testosterone propionate, this period is shorter than that for growth hormone. The necessity for a period of pretreatment suggests that the effects are produced through some metabolic change induced by the hormones, rather than by a direct effect of the hormones themselves.

Of all the hormone preparations used, those considered to be anabolic or nitrogen-sparing were the ones effective in preventing the ethionine fatty liver. This finding is consistent with the hypothesis (3) that the fatty change induced by ethionine in rat livers is causally related to some interference with protein metabolism.

SUMMARY

The effect of pretreatment with various hormones upon the level of liver lipides in ovariectomized Fisher rats receiving DL-ethionine intraperitoneally is reported.

Testosterone propionate, Δ^5-androstene-3β,17β-diol, methyltestosterone, and hypophyseal growth hormone prevented to a large degree the ethionine

[2] Farber, E., and Segaloff, A., unpublished work.

fatty liver and also decreased somewhat the liver lipide level in control animals. The degree of protection was not directly related to the androgenic potency of the androgens. Pretreatment with testosterone propionate for 7 days or longer, but not for 3 days, was effective. With growth hormone, the maximal degree of protection was observed only after 21 days of pretreatment. Δ^5-Androstene-3β,17β-diol produced a greater increase in the liver weight than did testosterone propionate.

Estradiol-17β, 11-deoxycorticosterone acetate, thyroid-stimulating hormone, prolactin, or crystalline zinc insulin were not protective. Cortisone acetate and ACTH intensified the fatty liver induced by ethionine. Sodium L-thyroxine decreased the liver lipide level in control and in ethionine-treated rats.

We would like to thank Dr. Sanford L. Steelman of the Armour Laboratories for generous supplies of the various growth hormone preparations, thyroid-stimulating hormone, ACTH, prolactin, and zinc insulin. The cortisone acetate suspension was supplied through the courtesy of Merck and Company, Inc. The sodium L-thyroxine was supplied through the courtesy of Dr. H. Roebling Knoch of the Smith, Kline and French Laboratories. The testosterone propionate, estradiol-17β, deoxycorticosterone acetate, methyltestosterone, and Δ^5-androstene-3β,17β-diol were supplied through the courtesy of Dr. Robert Gaunt, Mr. Fred Houghton, and Mr. Jack Cooper of Ciba Pharmaceutical Products, Inc. The ethionine was in part generously supplied by Dr. H. Tarver.

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HOMOGENTISATE OXIDASE OF LIVER*

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The liver enzymes which metabolize homogentisate[1] have been separated into an oxidizing fraction and a hydrolyzing fraction by Ravdin and Crandall (1). These workers isolated fumarylacetoacetate from the reaction catalyzed by the oxidizing fraction and found that this compound was hydrolyzed by the second fraction to the final products, fumarate and acetoacetate.

The present paper describes the preparation and properties of HG oxidase, apparently a single enzyme of a new type, and the reaction which it catalyzed. It oxidized HG not to FAcAc, as expected, but to another product. The probable identification of this product as 4-maleylacetoacetate, and its conversion to FAcAc by a specific cis-trans isomerase which uses glutathione as a coenzyme, will be described in later papers (2, 3).

Enzyme Assays

The oxidation of HG was followed by two methods in parallel, by oxygen uptake at 37° and by a spectrophotometric assay at 26–27°. The homogentisic acid used as the substrate (white crystals, m.p. 149°, corrected) was isolated as the lead salt from alkaptonuric urines (4). The reaction mixtures were identical for both assays. Each contained 1 ml. of 0.1 M 2,3,6-collidine buffer (5), pH 7.2, enzyme, and 5 to 8 μmoles of homogentisic acid (unneutralized to avoid inhibition by products of its non-enzymatic oxidation) in 3.0 ml. of total volume. Blank reactions without homogentisic acid were always run at the same time.

Assay by Oxygen Uptake—The reactions were run in Warburg respirometers at 37°, with air as the gas phase. The rate of oxygen uptake was linear until about 90 per cent of the substrate was oxidized, and the rate

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[1] In this paper the following abbreviations will be used: HG (homogentisate), FAcAc (fumarylacetoacetate), MAcAc (maleylacetoacetate), and GSH (glutathione).

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was proportional to the concentration of enzyme present. In order to detect the occurrence of any non-enzymic oxidation under the different experimental conditions, amounts of enzyme and substrate were chosen such that the reaction would be complete within an hour, and all the reactions measured were followed to completion. The occurrence of non-enzymic oxidation of HG was shown by a total oxygen uptake of more than the theoretical 1 molecule of oxygen per molecule of HG and by

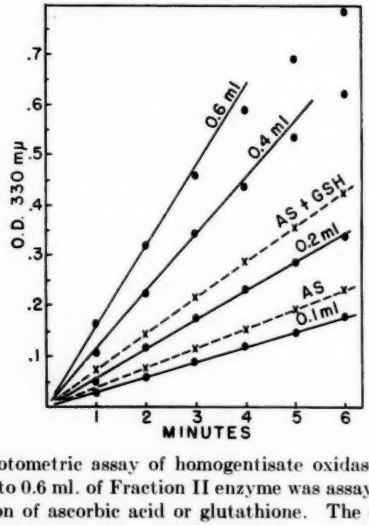

FIG. 1. Spectrophotometric assay of homogentisate oxidase at different enzyme concentrations. 0.1 to 0.6 ml. of Fraction II enzyme was assayed as described in the text, without addition of ascorbic acid or glutathione. The dotted lines show the reaction of 0.1 ml. of an ammonium sulfate-precipitated enzyme and its activation with 2.5 μmoles of glutathione. Ascorbic acid (AS) additions had the same effect in this preparation.

darkening of the reaction mixture. The results of such experiments were discarded.

Spectrophotometric Assay of HG Oxidation—Suitable enzyme preparations were assayed directly in the Beckman spectrophotometer by the change in the optical density at 330 mμ in a 1 cm. cell at 26–27°. The non-absorbing HG was converted by the reaction into a strongly absorbing diketo acid ($\epsilon_{330\ m\mu}$ = 13,500 at pH 7.2 for MAcAc). The optical density changed at a linear rate proportional to enzyme concentration, except at the highest levels of diketo acid accumulation (Fig. 1). The crude soluble liver fractions could not be assayed in this way because they accumulated no diketo acid. The removal of GSH by dialysis or alcohol precipitation of the enzyme was the most important factor in making a preparation suitable for

this assay. GSH addition was usually avoided in this assay because of its rôle in the further reaction of the diketo acid.

The rates of the reaction determined by the two assay methods paralleled each other in all the different enzyme preparations and were of approximately the same absolute magnitude. The molar rate of HG oxidation in the oxygen uptake assay was 2.2, 2.2, and 1.6 times that in the spectrophotometric assay in three different enzyme preparations. This difference could be attributed to the 10° difference in temperature between the two assays.

Preparations of Enzyme Fractions

Detailed observations were made only on the HG-oxidizing system from rat liver, although similar systems were found in the soluble fraction of rabbit, guinea pig, beef, pork, and horse liver homogenates. It was essential that all manipulations of the enzyme be carried out at 4° or lower, and at pH 7 or above, in order to achieve the two purposes of the preparations, the removal of GSH and the retention of enzymic iron. All preparations were stored at pH 7.5 and −9°.

Fresh rat livers were homogenized for 2 minutes in twice their volume of 0.9 per cent KCl. The soluble fraction (Fraction I), obtained by 15 minute centrifugation at 12,000 × g, contained all the HG oxidase activity of the original homogenate (approximately 200 μl. of O_2 uptake in 10 minutes per ml.). This fraction was adjusted to pH 7 and cooled to 0°. 0.5 volume of 95 per cent ethyl alcohol was added slowly and the mixture immediately centrifuged. The precipitate was drained well and then resuspended in fresh 32 per cent alcohol at 0° equal in volume to the supernatant fraction removed. After another centrifugation the precipitate was dissolved in water (one-half the volume of the original Fraction I), adjusted to pH 7.5, and dialyzed against water for 2 hours with efficient stirring. This was then frozen at −9° (Fraction II). When it was thawed at least 24 hours later, a large inactive precipitate was centrifuged off. The fraction contained about one-half the activity, and had about twice the specific activity, of Fraction I. There was a sufficient degree of functional purification to cause the accumulation of MAcAc. The enzyme at this stage was stable at −9° for several months.

Part of the activity in Fraction II could be precipitated between 50 and 70 per cent saturation with ammonium sulfate adjusted to pH 7.5 with ammonia. The activity in this preparation did not survive the acidification incidental to dialysis and could be preserved only for several days. The preparation had a somewhat higher specific activity than Fraction II and required activation by reducing agents (see below).

Properties of Homogentisate Oxidase

HG Oxidase Reaction—The reactions catalyzed by the different enzyme preparations are described in Table I. The enzyme preparations all took up approximately 1 molecule of oxygen per molecule of substrate, regardless of which product was formed. The reactions of all the many enzyme preparations studied here were followed to completion, and none showed evidence of only 1 atom of oxygen taken up per molecule of HG, although the preparations differed in source, age, and previous treatment, and many were assayed in the presence of various inhibitors. In particular, neither the absence of phosphate nor the use of borate buffer resulted in the uptake of 0.5 molecule of oxygen per molecule of substrate, as reported by Suda and Takeda (6), although the enzyme was soon inactivated in borate by acidification of the reaction mixture unless the pH was controlled by other means.

TABLE I

Reaction of Homogentisate in Different Liver Fractions

The indicated amounts of homogentisate were oxidized to completion as described for the oxygen uptake assay. CO_2 was not formed in the reaction, as shown by identical manometric changes with and without NaOH in the center wells. CO_2 evolution from bicarbonate-CO_2 buffer was determined by difference with the O_2 uptake measured in collidine buffer. Acetoacetate was determined by aniline citrate decarboxylation in 10 minutes (7). Maleylacetoacetate was determined spectrophotometrically at 330 mμ (2).

	HG added	O_2 uptake	O_2/HG	MAcAc	AcAc
	μmoles	*μmoles*		*μmoles*	*μmoles*
Fraction I	6.3	5.5	0.87	0	5.0
" II*	5.0	4.4	0.88	3.6	0.8
" " + supernatant†	5.0	4.5	0.90	0	4.4
$(NH_4)_2SO_4$ ppt.	6.3	6.3	1.00	5.1	0‡
		CO_2 evolved	CO_2/HG		
In bicarbonate buffer					
Fraction II§	4.1	4.3	1.05	4.0 (3.6)‖	0‡
" "	4.1	4.0	1.02	3.8 (4.0)‖	0‡
" " + supernatant†	4.1	7.8	1.90	0	4.3

* Once precipitated with 32 per cent alcohol.

† 0.2 ml. of supernatant fraction from the alcohol precipitation of Fraction II.

‡ Essentially none. The exact amount was indeterminate in the presence of the slowly decarboxylating diketo acid.

§ Precipitated and washed with 32 per cent alcohol.

‖ Determined by aniline citrate decarboxylation in 70 minutes.

Table I also indicates that acetoacetate was a product of the reaction in the crude extract (Fraction I), but that the yield of acetoacetate was decreased after one alcohol precipitation of the enzyme (Fraction II) and eliminated by further treatment of the enzyme. An intermediate which was decarboxylated by aniline citrate (7) more slowly than was acetoacetate replaced it as the reaction product. The addition of the supernatant fraction left from the alcohol precipitation of Fraction II restored the formation of acetoacetate without changing the oxygen consumption of the reaction. This supernatant fraction after dialysis lost the ability to form acetoacetate from the intermediate unless GSH was added, although the same dialyzed fraction without GSH formed acetoacetate from FAcAc. This observation formed the basis for an enzymic differentiation between the new product and FAcAc (3).

In the last experiments of Table I, the accumulated precursor of acetoacetate was a dicarboxylic acid, since 1 molecule of CO_2 was liberated from bicarbonate buffer for each molecule of HG oxidized to the intermediate. The conversion of this dicarboxylic acid intermediate to acetoacetate in the presence of the undialyzed supernatant fraction was associated with the liberation of a second molecule of CO_2. This indicated the formation of an additional acid group which would be expected from the enzymic hydrolysis of FAcAc to fumarate and acetoacetate, the demonstrated products of this reaction (1). However, FAcAc was not accumulated by any of the enzyme preparations. Most preparations of the Fraction II precipitated once with alcohol were in fact able to hydrolyze FAcAc. The new dicarboxylic acid intermediate which was accumulated was a diketo acid distinguished from FAcAc by its lack of any reaction with the dialyzed supernatant fraction from Fraction II which hydrolyzed FAcAc, and by its absorption spectrum, which was used to measure it in the experiments of Table I (2).

Rôle of Iron in HG Oxidase—The HG oxidase did not display the usual properties of known iron-containing oxidative enzymes. Prepared as described and kept at a pH of 7 or above, it was not significantly activated by the addition of Fe^{++}. Acidification to pH 5, which was earlier found to inactivate the enzyme (8), resolved it so that activation with Fe^{++} occurred as has been described (9), but the original activity could be only partially restored and hence such treatment was avoided in the preparation procedures. The HG oxidase reaction did not form hydrogen peroxide detectable by the coupled oxidation of ethyl alcohol with catalase (10), and the intermediary formation of peroxide did not occur as in the liver tryptophan-peroxidase-oxidase system (11). The latter type of reaction was excluded because no requirement for peroxide was revealed by partial purification of the enzyme, catalase did not cause inhibition, and peroxide generated by glucose oxidase during the reaction did not activate the re-

action. CO in the dark did not inhibit the enzyme (Table II) as it does the iron-porphyrin proteins which react with oxygen.

The effects of several other compounds which react with iron are given in Table II to define further the rôle of iron in the HG oxidation reaction. The complete inhibition of oxygen uptake which was caused by α,α-dipyridyl was paralleled by complete inhibition of diketo acid or acetoacetate formation in the same experiments. These results provided no evidence for a participation of iron in reactions later than the first oxidation step,

TABLE II

Inhibition of Homogentisate Oxidase by Compounds Reacting with Iron

These results were obtained in oxygen uptake assays. The compounds tested were in contact with the enzyme 10 minutes before the substrate was added. Similar results with all compounds were obtained in the spectrophotometric assay.

Compound	Concentration	Inhibition
	M	*per cent*
α,α-Dipyridyl	5×10^{-3}*	100
"	1.7×10^{-3}*	80
Na azide	3.3×10^{-3}	83
NaCN	3.3×10^{-3}	27
$CO–O_2$	80–20% (Dark)	0
Phosphate	3×10^{-2}†	28–40
Pyrophosphate	3×10^{-2}†	44
Cysteine	3×10^{-3}	73‡
Cystine	4.5×10^{-3}	53‡
Cysteic acid	4.5×10^{-3}	15

* The solution was colorless until the substrate was added; then it immediately turned red.

† Compared with the collidine-buffered reaction. The inhibition by phosphate was variable.

‡ Not reversed by 0.005 M GSH added 5 minutes after the inhibitor.

as was suggested by Schepartz (9). We observed an excess of oxygen uptake over acetoacetate formation such as he reported only when the diketo acid was accumulated as a result of the lack of GSH or when non-enzymic oxidation of HG occurred. The latter reaction was seen only in certain preparations with low activity, those containing heat-inactivated enzyme, or in the presence of an excess of metal salts or of complex-forming agents. In addition to the inhibition by α,α-dipyridyl, which reacts with ferrous iron, the reaction was also inhibited by azide and cyanide, which characteristically react with ferric iron. These results suggested a valence change of iron during the reaction. The effects of pyrophosphate and even of phosphate on the reaction emphasized the availability of the iron, already suggested by the easy resolution of the enzymic iron.

Cysteine and cystine inhibited HG oxidation and were at the same time rapidly oxidized in the system. Cysteic acid, whose maximal known effect is given in Table II, was considerably less inhibitory. Either chelation of the enzyme iron by cysteine or irreversible oxidation of groups on the enzyme, perhaps by peroxide, was a more likely cause of the cysteine inhibition than a reaction of cystine with enzyme thiol groups, since GSH did not reverse the cysteine or cystine inhibitions.

TABLE III

Inhibition and Activation of Homogentisate Oxidase As Sulfhydryl Enzyme

The substances to be tested were added 10 minutes before the substrate in the oxygen uptake assay and 3 minutes before the substrate in the spectrophotometric assay. All effects were demonstrable by both assays. The activations (+) were all measured in enzymes which had been precipitated with ammonium sulfate at pH 7.5, but were occasionally observed also in Fraction II preparations.

Substance tested	Concentration or amount	Assay	Difference from control activity
			per cent
$HgSO_4$	3×10^{-5} M	O_2	−100
"	1.5×10^{-5} M	"	−63*
"	6×10^{-6} M	"	0
p-Chloromercuribenzoate	3×10^{-5} "	"	−66*
H_2O_2 from glucose oxidase	0.05 μmole per 10 min.	"	−65†
Benzoquinoneacetic acid	2.9×10^{-4} M	Spectrophotometric	−84
Ferricyanide	1×10^{-2} M	O_2	−100
Ascorbic acid	8×10^{-4} "	Spectrophotometric	+83
" "	8×10^{-4} "	O_2	+13
Glutathione	8×10^{-4} "	Spectrophotometric	+51
"	8×10^{-4} "	O_2	+46
Coenzyme A	0.3 mg.	"	+38
Mercaptoacetic acid	8×10^{-4} M	Spectrophotometric	+38
Denatured liver protein‡	5 mg.	O_2	+64

* Inhibition completely reversed by glutathione (10^{-3} M) added 5 minutes after the substrate.

† Inhibition was prevented by the earlier addition of 1 mg. of crystalline catalase but was not reversible.

‡ Precipitated between 60 and 70 per cent alcohol from dialyzed rat liver homogenate and heated at 60° for 5 minutes. Positive nitroprusside test.

Essential Thiol Groups of HG Oxidase—The inhibition of the enzyme by Hg^{++} and by *p*-chloromercuribenzoate, both reversed by GSH, and the irreversible inhibitions by other reagents given in Table III demonstrated the presence of essential thiol groups on the enzyme. This finding can explain the previously observed inhibitions of this enzyme by benzoquinoneacetic acid, several other quinones, and maleate (9).

Certain enzyme preparations (notably the ammonium sulfate fractions) required addition of reducing agents for full activity. This requirement was at first overlooked because of the inhibition caused by cysteine. The activation of HG oxidase by ascorbic acid and several thiols is shown in Table III. This activation was more readily demonstrated in the oxygen uptake assay than under the milder conditions and shorter reaction period of the spectrophotometric assay. Examples from both assays are given, including activation by GSH in the spectrophotometric assay in which the activation could be demonstrated only in a preparation nearly free of the enzymes which removed the diketo acid (see also Fig. 1). Activation by a thiol-containing protein emphasized the lack of specificity of this effect.

A small activation of the oxidation of HG to acetoacetate was reported by Williams and Sreenivasan, who suggested that it was a specific effect of ascorbic acid (12). However, they did not test GSH or the other effective reducing agents in this reaction. Ascorbic acid also activated resolved HG oxidase by reducing ferric to ferrous iron (6). Ferrous iron did not activate the preparations used in the present experiments. The activations observed were most probably the result of protection of thiol groups of the enzyme from oxidation during the reaction.

DISCUSSION

The most important factors in obtaining active and stable HG oxidase preparations which accumulated the new product of the reaction were the removal of GSH, avoidance of resolution of the enzymic iron, and the addition of reducing agents such as ascorbic acid when these were required.

The reaction catalyzed by these preparations of HG oxidase showed the stoichiometry expected for the formation of a compound similar to FAcAc, which has been isolated from similar preparations. 1 molecule of oxygen was taken up and 1 molecule of CO_2 was displaced from bicarbonate, per molecule of HG oxidized. The accumulated compound was not FAcAc, but was converted to acetoacetate (and presumably fumarate) by the fraction of liver separated from the oxidase.

The oxidation of HG with 1 molecule of oxygen to the new diketo acid appeared to be the result of the action of a single enzyme, to which we restrict the name HG oxidase. The enzyme can be partially defined in terms of its reaction, the nature of its contained iron, and its essential thiol groups. These properties distinguish it from other types of known enzymes with the possible exception of two bacterial enzymes, pyrocatecase and protocatechuic acid oxidase, whose similarities with the present enzyme have already been recorded by Stanier and Ingraham (13). It may be that HG oxidase represents a new type of non-porphyrin, iron-containing oxidative enzyme. An association of the iron with the protein thiol groups in this enzyme has been suggested by recent experiments of Crandall (14).

SUMMARY

1. Homogentisate oxidase, prepared from liver by alcohol precipitation, formed a new diketodicarboxylic acid of the same oxidation level as fumarylacetoacetate. The accumulation of this initial product of the oxidation was the basis of a spectrophotometric assay of the enzyme reaction.

2. The participation of iron in this reaction was confirmed. There was evidence that the iron behaved unlike an iron porphyrin and changed valence during the reaction.

3. The enzyme possessed essential sulfhydryl groups, and certain preparations were activated non-specifically by ascorbic acid, glutathione, and other thiol compounds except cysteine.

Dr. Charles H. DuToit, Massachusetts General Hospital, and Dr. James Patterson, New England Center Hospital, kindly made available to us urine collections from their alkaptonuric patients as a source of homogentisic acid. The technical assistance of Miss Anita Aspen is gratefully acknowledged.

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THE PROPERTIES OF MALEYLACETOACETATE, THE INITIAL PRODUCT OF HOMOGENTISATE OXIDATION IN LIVER*

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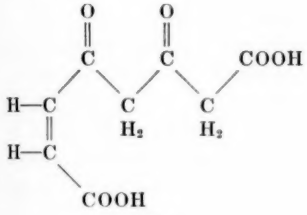
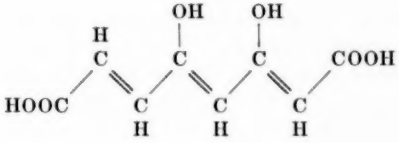
A new oxidation product of homogentisate, formed by a preparation of liver homogentisate oxidase (1), is described in this paper. During attempts to isolate this new intermediate, it was readily converted to fumarylacetoacetate, a compound with a different absorption spectrum previously isolated from this reaction by Ravdin and Crandall (2). The properties of the new intermediate and the identification of maleic acid formed from it by hydrolysis suggested that it was maleylacetoacetate, the cis isomer of fumarylacetoacetate. Maleylacetoacetic acid (I) and fumarylacetoacetic acid (II) existed in acidic solutions as their keto and enol forms, respectively, which accounted for their different absorption spectra.



(I)



(II)

EXPERIMENTAL

Formation of New Intermediate (Maleylacetoacetate) (MAcAc)—Homogentisate oxidase, prepared from liver by 32 per cent ethanol precipitation and by reprecipitation to remove glutathione (GSH), was used to form the

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† Public Health Service Research Fellow of the National Institutes of Health.

new intermediate by the complete oxidation of homogentisate, as previously described (1). The reactions were carried out in Warburg vessels with oxygen as the gas phase. About 5 mg. of enzyme protein were used per vessel to catalyze the oxidation of 6 μmoles of homogentisate, with the uptake of 6 μmoles of oxygen, in 15 minutes. Longer reaction times were avoided in order to minimize degradation of the product to fumarylacetoacetate (FAcAc) and subsequent hydrolysis by FAcAc hydrolase, which was present in all the enzyme preparations. When the oxygen uptake of the reactions stopped, the flasks were promptly chilled. The pooled contents of a number of these, and of the control reaction mixtures without homogentisate, were deproteinized with 0.1 volume of 20 per cent metaphosphoric acid. The filtrates were immediately made 0.1 N with respect to NaOH and stored at −9°.

The amount of the new intermediate that was accumulated depended upon the completeness with which glutathione had been excluded from the reaction mixture. Freshly made filtrates contained no FAcAc. Some FAcAc was formed in the stored solutions, and this was removed when necessary by treatment at neutral pH with FAcAc hydrolase (see below).

Preparation of Fumarylacetoacetate—The metaphosphoric acid filtrates described above were kept at 10° without addition of NaOH. After 5 days the non-enzymic isomerization of the new intermediate to FAcAc was nearly complete. These solutions of FAcAc were kept at 4° for no more than 2 weeks. The older solutions of FAcAc contained appreciable amounts of a compound with an absorption spectrum similar to that of FAcAc, but which was not hydrolyzed by FAcAc hydrolase.

Determination of FAcAc and New Intermediate—The two compounds were identified and determined in every instance both spectrophotometrically from their absorption spectra at pH 1 and 13 and by their hydrolysis with specific enzymes. FAcAc absorbed strongly in both acid and alkaline solutions, as previously reported (2), and the new intermediate absorbed strongly in alkaline solution only. Both compounds had significant absorptions at pH 7.5. The absorption spectra of both compounds at pH 1 and 13 are given in Fig. 1.

Specific enzymes for the determination of the new intermediate and FAcAc were both present in the supernatant fraction left after the alcohol precipitation of the homogentisate oxidase (1). If this preparation was well dialyzed, it hydrolyzed only FAcAc. When GSH, the coenzyme of MAcAc isomerase (3), was added, the preparation hydrolyzed the new intermediate as well. Hydrolysis was determined at pH 7.5 by the disappearance of the optical density of the two compounds at 330 mμ. FAcAc was also hydrolyzed by FAcAc hydrolase (2) and by the enzyme of Connors and Stotz (4).

Table I gives the molar extinction coefficients of the new intermediate and FAcAc at 330 mμ and the formulas which were used to calculate from the optical density measurements the concentrations of the two compounds in mixtures. For the purpose of determining the extinction coefficient of the new intermediate, its concentration was assumed to be equal to that of the homogentisate oxidized. Known amounts of homogentisate were completely oxidized in six different enzyme preparations which accumu-

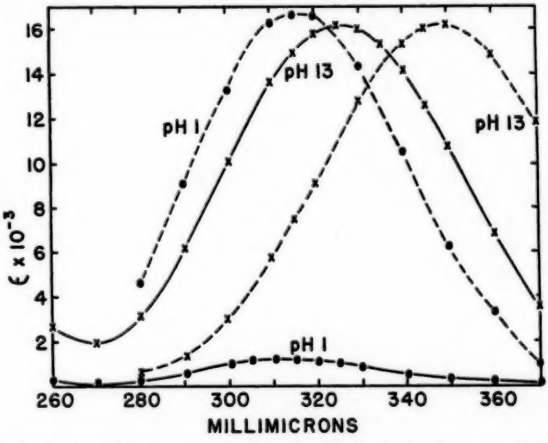

FIG. 1. Dash line, absorption curves of fumarylacetoacetate, and solid line, the new intermediate (MAcAc), each at ●, pH 1, and ×, pH 13. The extinction coefficients are based on the 330 mμ values given in Table I. The curves for the new intermediate were determined directly on a freshly prepared sample, and those for FAcAc were corrected by subtraction of a small amount of absorption left after FAcAc hydrolase treatment of the samples. The absorption maxima at pH 13 were 326 and 349 mμ for the new intermediate and FAcAc, respectively, and 317 mμ for FAcAc at pH 1.

lated maximal amounts of the new intermediate. The optical densities at pH 13, 7.5, and 1 of the product formed were immediately determined. From the oxygen uptakes and the acetoacetate determinations in these reactions the accumulation of the new intermediate appeared to be at least 90 per cent complete. The extinction coefficients of our preparations of FAcAc agreed closely with those determined by Ravdin and Crandall for solutions of the isolated silver salt (2), although the absorption spectra differed slightly as a result of our correction for the absorption of the impurity which formed in aged solutions and which was not hydrolyzed by FAcAc hydrolase. Fresh preparations of both FAcAc and the new inter-

mediate were completely hydrolyzed enzymically to give solutions without significant absorption above that of the blanks.

Chemical Properties of New Intermediate—The new product and FAcAc were very similar, despite their different absorption curves and the reaction of only the latter with FAcAc hydrolase. Previous observations on the enzymic oxidation of homogentisate to the new compound showed that in this reaction 1 molecule of oxygen was consumed, and in the presence of bicarbonate 1 molecule of CO_2 was evolved, per molecule of homogentisate (1). The same result would be expected if the product of homogentisate oxidation was FAcAc. The new product also was decarboxylated with aniline citrate (5) in 70 minutes, at the same rate as was FAcAc. Both

TABLE I

Molar Extinction Coefficients of Diketo Acids

The molar extinction coefficients (ϵ, the optical density of a 1 M solution 1 cm. in depth) at 330 mμ are given with standard errors of the mean. D_1 and D_{13} are the observed optical densities at 330 mμ in 1 cm. cells in 0.1 N HCl and 0.1 N NaOH, respectively, of mixtures of the two diketo acids. The concentration of each diketo acid was calculated with the formulas given.

	$\epsilon_{330\ m\mu} \times 10^{-2}$			μmoles per ml.
	pH 1	pH 7.5	pH 13	
New intermediate (MAcAc)	1.2 ± 0.05	14.0 ± 0.6	16.0 ± 0.6	$(D_{13} - D_1)/14.8$
Fumarylacetoacetate	13.5*	10.7 ± 0.15	13.5*	$0.08\ D_1 - 0.006\ D_{13}$

* From data of Ravdin and Crandall (2).

FAcAc and the new compound gave the reaction for diketones with *o*-phenylenediamine (6, 2). Both gave the same rates of color development, final color intensities, and absorption curves of the colors formed in this reaction. Also typical of a β-diketone (7, 4) was the weak light absorption at pH 1 and strong absorption at pH 13 of the new intermediate (Fig. 1). (Ravdin and Crandall pointed out that the high absorption of FAcAc in acid solution was atypical of a β-diketone (2). The reason for this absorption will be discussed below.) There was no evidence for the formation or hydrolysis of a lactone when the pH of the new intermediate was changed, since the acid and alkaline curves of the new intermediate were interconvertible within 30 seconds, and since no hydroxamic acid (8) was formed in neutralization of acid solutions of the new intermediate or FAcAc in the presence of hydroxylamine. These findings indicated that the new intermediate was a β,δ-diketodicarboxylic acid very similar to FAcAc.

Conversion of New Intermediate to FAcAc—The conversion of the new intermediate to FAcAc occurred during the attempted isolation of the new compound by silver precipitation by which FAcAc had been isolated (2), as well as during a number of other procedures. This conversion occurred in solutions merely on standing, as illustrated in Fig. 2. It was rapid in acid solutions, more so at higher temperatures, and it was nearly stopped in alkaline solution. The reaction in acid solutions was not accelerated by irradiation with sunlight in quartz vessels with or without traces of I_2, nor was it accelerated by various heavy metal salts. At neutral pH the slow isomerization was slightly accelerated by glutathione. No oxidation,

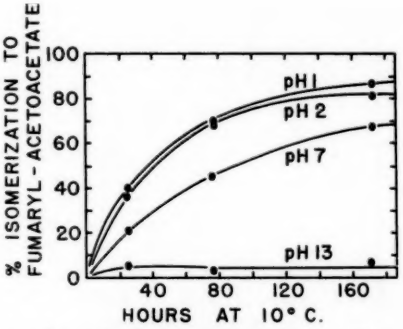

FIG. 2. Spontaneous isomerization of the new intermediate to fumarylacetoacetate. Samples of the same enzyme filtrate were adjusted to the different pH values, and serial spectrophotometric determinations were made of the two compounds present. No corrections were made for the small loss of diketo acid in the pH 13 sample (about 10 per cent).

phosphorolysis, or other chemical reaction could be associated with the conversion. The reverse reaction, from FAcAc to the new intermediate, did not occur.

Reduction of New Intermediate and FAcAc to Succinylacetoacetate—The presence of a double bond in the new intermediate was indicated by its reduction by 1 molecule of H_2 in the presence of Pd to a substance which had the typical absorption spectra of a diketo acid and which was indistinguishable from the substance formed by the reduction of FAcAc. Two preparations of the reduced product of the new intermediate had molar extinction coefficients, at 295 mμ in alkali, of 16.9 and 20.3 $\times$ 10^3. Ravdin and Crandall (2) published the very similar absorption curve of a reduced product of FAcAc, assumed to be succinylacetoacetate, which had a molar extinction coefficient of about 20.5 $\times$ 10^3 at its absorption maximum at 295 mμ. The absorption curve of what was apparently one compound,

formed by the reduction of a mixture of the new intermediate and FAcAc in the same solution, is illustrated in Fig. 3. This reduction product was completely hydrolyzed by FAcAc hydrolase in the absence of glutathione.

Additional evidence for the presence of an olefine group in the new intermediate was its ability to decolorize $KMnO_4$ in metaphosphoric acid solution, in the Dickman method for the determination of olefine compounds (9). FAcAc reacted in this determination at the same rate and to the same

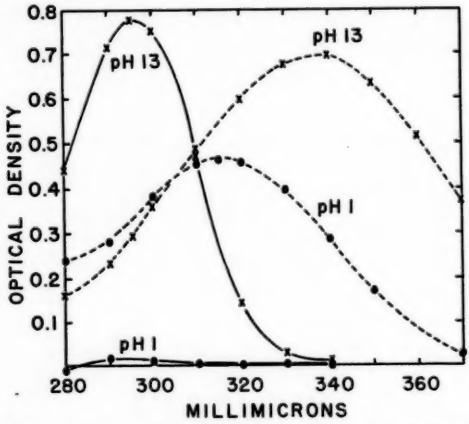

FIG. 3. Absorption curves at ●, pH 1, and ×, pH 13, of 1:60 dilutions of a mixture of the new intermediate (MAcAc) and fumarylacetoacetate before (dash line) and after (solid line) reduction by H_2 (Pd) to succinylacetoacetate. The mixture of diketo acids, derived from 10.0 μmoles of homogentisate, took up 10.3 μmoles of H_2 in 10 minutes at 37°. The reaction mixture contained 3.4 μmoles of the new intermediate and 5.0 μmoles of FAcAc by analysis, and 0.5 mg. of 10 per cent palladium charcoal in a total volume of 3 ml. of 0.7 per cent metaphosphoric acid.

extent as did the new intermediate. Both compounds also took up Br_2 and gave unsaturated acids on hydrolysis in alkali (see below).

Degradation of New Intermediate and FAcAc—Filtrates containing these two compounds were hydrolyzed for 1 hour in alcoholic KOH to their component unsaturated dicarboxylic acids under conditions which avoided any isomerization of maleic and fumaric acids. The unsaturated acids were obtained in yields amounting to 20 or 30 per cent of the amounts of diketo acid treated, by the use of IRC-50 and IR-4B Amberlite columns. The acids were identified chromatographically on paper sprayed with 0.01 N $KMnO_4$ in 0.5 per cent metaphosphoric acid (Dickman's reagent (9)) and with bromocresol green. Three different formic or acetic acid-alcohol mixtures were used for chromatography which were so chosen that

maleic acid ran slower in two of the mixtures and faster in the third than did fumaric acid. Blank enzyme reaction filtrates (no homogentisate) yielded no acids on chromatography after the hydrolysis. FAcAc-containing filtrates after hydrolysis yielded on chromatography a single spot which was not apparent before hydrolysis and which was identified as fumaric acid in the three solvents. Freshly prepared filtrates of the new intermediate yielded after hydrolysis a main component identified as maleic acid in all three solvents, plus traces of fumaric acid. The small amounts of fumaric acid found with the maleic acid from these solutions of the new

TABLE II

Bromine Titrations of Two Diketo Acids in Metaphosphoric Acid

All the values are in micromoles. The bromine uptakes are the means of triplicate determinations, each of which was within 5 per cent of the mean. They were obtained by the addition of sufficient Br_2 within a 2 minute period to bleach added methyl orange (10). The diketo acids did not release I_2 from KI after treatment with Br_2 in the usual enol method (11). The Br_2 uptake per molecule of fumarylacetoacetate is calculated by difference on the basis of an uptake of 1 molecule of Br_2 per molecule of the new intermediate (MAcAc) in the solutions.

Sample No.	Diketo acids in samples		Bromine uptake		
	FAcAc	MAcAc	Total	Per μmole of total diketo acids	Calculated, per μmole of FAcAc
1	0.4	8.4	11.6	1.3	
2	2.2	8.8	16.0	1.5	3.3
3	7.6	3.4	28.0	2.5	3.2
4	6.4	2.2	24.2	2.8	3.4

intermediate could have been formed enzymically during the preparation of the new intermediate.

Determination of Enol Groups in FAcAc—A marked difference was found in the amount of Br_2 taken up within 2 minutes by acid solutions of the new intermediate and of FAcAc. The results of these titrations are given in Table II. Solutions containing mainly the new intermediate consumed little more than 1 molecule of Br_2, and solutions containing mainly FAcAc consumed nearly 3 molecules of Br_2, per molecule of total diketo acids. Assuming an uptake of 1 molecule of Br_2 by each molecule of the new intermediate present in each solution (the best measurement was 1.3 molecules of Br_2 per molecule of new intermediate), the calculated Br_2 uptake per molecule of FAcAc was about 3.3 molecules for three different solutions in which FAcAc accounted for 20 to 75 per cent of the diketo acid present. Any isomerization of the new intermediate or conversion of keto to enol groups during the short reaction period would have increased the measured

Br_2 uptake. Regardless of this possibility, which can be eliminated only by study of the pure compounds by other methods, it was clear that the olefine group of either diketo acid could account for the reaction of 1 molecule of bromine, and that FAcAc possessed, in addition, approximately two reactive enol groups per molecule which the new intermediate did not have.

DISCUSSION

Ravdin and Crandall, although they isolated a trans isomer (2), mentioned the probability that the first product formed by the opening of the homogentisate ring would be an unsaturated cis compound. Both chemical and biological oxidations of the aromatic ring of benzene, for example, produced *cis-cis*-muconic acid (12, 13). The evidence for the identification of the new intermediate as the cis isomer, maleylacetoacetate, consists of its chemical similarity to FAcAc, the chromatographic identification of maleic acid formed from it by hydrolysis, its ready but irreversible conversion to FAcAc (the direction of isomerization is usually cis to trans), and the reduction of the olefine bond of both compounds with H_2 to form what was apparently a single product. In addition, the absorption maximum at pH 13 of the new intermediate (326 mμ) was lower than that of FAcAc (349 mμ). This is the characteristic relation of the cis to the trans isomer among the carotenoids (14) and among a number of olefinic acids examined by us.

The inability of FAcAc hydrolase to act on MAcAc cannot be explained from the present knowledge of this enzyme. The possible specificity of the hydrolase for a particular geometrical isomer or for highly enolized substrates was not tested in the previous studies of its action on a number of simple diketo acids (7, 4). Additional studies will be required to determine the basis of this specificity.

The major difference between MAcAc and FAcAc which must be explained by the geometrical isomerism was the difference in the acid absorption spectra. The absorption of FAcAc in acid solutions could be attributed to its enol form, in view of the similarity of this absorption to those of the enolate forms of both MAcAc and FAcAc in alkali. The demonstration by Br_2 titration that FAcAc did exist in an enol form in acid solution and that MAcAc existed in the keto form adequately accounted for the different absorption spectra of the two compounds on this basis. However, an explanation is needed to account for the high and unique degree of enolization shown by the trans isomer in aqueous acid solution. An unusual stabilization of the enols of both diketo acids can be assumed to result from the conjugation of the five double bonds in the enol forms of these compounds, and the existence of the enol form of FAcAc can be attributed to

this effect. The enol form of MAcAc must be regarded as a "hindered cis" compound, as defined by Pauling (15). The difference between the two compounds must therefore reside in this hindrance of the enol group adjacent to the cis olefine bond, in consequence of which only the keto form of MAcAc exists in acid solution.

No FAcAc was ever observed in our enzyme reaction mixtures examined shortly after the end of the reaction. Our preparations of homogentisate oxidase contained small amounts of FAcAc hydrolase which prevented the accumulation of any FAcAc. It would appear possible that MAcAc accumulated also as the product of the reaction in the experiments of Ravdin and Crandall, who used enzyme preparations similar to ours. The absorption spectrum of their initial product, which would have determined its nature, was not described. If they did accumulate MAcAc instead of FAcAc, the MAcAc would have isomerized in the course of their isolation procedure to the FAcAc they isolated. There is no doubt that the isolated product was FAcAc. Many of the properties of FAcAc described by Ravdin and Crandall were confirmed during this study, and an independent confirmation of its structure was obtained by its hydrolysis in alkali to fumarate. The proof that FAcAc itself is an enzymically formed intermediate in homogentisate metabolism, however, will depend upon the demonstration of its enzymic formation from MAcAc.

SUMMARY

1. The primary product of homogentisate oxidation by a liver enzyme was identified as an unsaturated β,δ-diketodicarboxylic acid similar to fumarylacetoacetate. It was different from fumarylacetoacetate in its absorption spectra, and it was not hydrolyzed by fumarylacetoacetate hydrolase. The new intermediate spontaneously changed into fumarylacetoacetate in acid but not in alkaline solution.

2. The chromatographic identification of maleic and fumaric acids, respectively, after alkaline hydrolysis of the new intermediate and of fumarylacetoacetate, was consistent with the identification of the new intermediate as 4-maleylacetoacetate, the cis isomer of fumarylacetoacetate. Additional evidence supported this identification.

3. The difference in the absorption spectra of the diketo acids in acid solution was attributed to the existence of fumarylacetoacetic acid in its enol form and of maleylacetoacetic acid in its keto form.

We wish to thank Dr. Burnett M. Pitt for making the bromine titrations given in Table II. We are indebted to Dr. Ruth Hubbard and to Dr. Robert I. Gregerman for helpful discussions about the relationship of geometrical isomerism to absorption spectra.

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THE IDENTIFICATION OF 5-HYDROXY-3-INDOLEACETIC ACID IN NORMAL URINE AND A METHOD FOR ITS ASSAY*

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The wide-spread distribution of 5-hydroxytryptamine (serotonin) in mammalian tissues which are rich in monoamine and aldehyde oxidases suggested that oxidation to 5-hydroxyindoleacetic acid (5HIAA) might be a major route of its metabolism. Experiments previously reported (1) have indicated that this is indeed the case. Studies described in this paper show that 5HIAA is a normal constituent of mammalian urine. Details of a colorimetric method for its determination are presented.

EXPERIMENTAL

Reagents—

1-Nitroso-2-naphthol. 0.1 per cent solution in ethanol.

Nitrous acid reagent. To 5 ml. of 2 N H_2SO_4 is added 0.2 ml. of 2.5 per cent $NaNO_2$. The reagent should be prepared fresh daily.

Ethyl ether, reagent grade, was washed once with a dilute solution of ferrous sulfate to destroy peroxides and twice with water.

0.5 per cent 2,4-dinitrophenylhydrazine in 2 N HCl.

0.5 M phosphate buffer, pH 7.0.

Identification of 5HIAA in Human Urine—In a typical experiment, 90 ml. of urine were adjusted to about pH 3 by the dropwise addition of concentrated HCl and extracted twice with 400 ml. portions of $CHCl_3$ to remove indoleacetic acid. The $CHCl_3$ was discarded and the acidified urine after saturation with NaCl was extracted with 250 ml. of ether. The apparent 5HIAA was returned to an aqueous phase by shaking the ether solution with 50 ml. of phosphate buffer, pH 7.0. The latter phase was acidified and extracted with 250 ml. of fresh ether. The presence of 5HIAA

* Part of the material contained in this paper was taken from a thesis submitted by Herbert Weissbach to the Graduate School of George Washington University in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry.

in the ether solution thus obtained was shown by the experiments described below.

Identification by Paper Chromatography—Portions of the ether extract containing 10 to 30 γ of apparent 5HIAA were evaporated to approximately 0.2 ml. for transfer to Whatman No. 1 paper for chromatography. To avoid excessive destruction of 5HIAA the sample was not evaporated completely to dryness. When chromatograms were developed with *n*-propanol-water (5:1), an R_F of 0.75 was observed for both the 5-hydroxyindole compound isolated from urine and authentic 5HIAA. With *n*-propanol-1 N ammonia (5:1) as a developing solvent, an R_F of 0.27 was observed

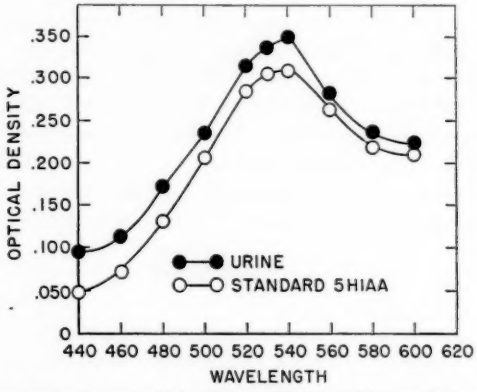

FIG. 1. Absorption spectra of nitrosonaphthol reaction products with synthetic 5HIAA and that obtained from urine. 1 ml. of extract containing approximately 20 γ of 5HIAA was treated with 0.5 ml. each of nitrosonaphthol and nitrous acid reagent as described in the text.

for both substances. For visualization, chromatograms were sprayed with the nitrosonaphthol and nitrous acid reagents or with *p*-dimethylaminobenzaldehyde, as described previously (2). 5-Hydroxyindoles appear as violet spots on a faint yellow background with the nitrosonaphthol reagent and as blue spots with *p*-dimethylaminobenzaldehyde.

Comparison of Absorption Spectra of Nitrosonaphthol Reaction Products—A portion of the ether extract containing approximately 40 γ of 5HIAA was extracted with 2 ml. of the phosphate buffer. A 1 ml. aliquot of the buffer solution was treated with the nitrosonaphthol and nitrous acid as described in the analytical procedure below, and an authentic sample of 5HIAA was treated in the same manner. The absorption spectra of the two solutions (Fig. 1) were essentially the same.

Reaction with nitrosonaphthol indicates a phenolic compound. The

formation of a violet chromophore is characteristic of 5-hydroxyindole compounds, as shown previously (2). For example, tryptamine and various 7-hydroxyindoles do not respond. Of over twenty phenolic compounds tested, only *p*-hydroxyacetanilide reacted to give this color.

Distribution between Solvents—A comparison of the distribution of 5HIAA and apparent 5HIAA between ether and water at various pH values, according to the procedure of Brodie, Udenfriend, and Baer (3), provided further confirmation of their identity. It is clear from the data of Table I that the nitrosonaphthol-reacting material obtained from urine contains

TABLE I

Distribution of 5HIAA and Apparent 5HIAA between Water and Ether at Various pH Values

The apparent 5HIAA was contained in the final ether extract of urine described in the text. 15 ml. aliquots of this solution and of a 5HIAA solution in ether were shaken with 1.5 ml. volumes of buffer, and 1 ml. portions of the aqueous extracts were assayed by the nitrosonaphthol color reaction. The fraction of the compounds extracted at various pH values is expressed as the ratio of the amount of compound in the aqueous phase to the total compound.

pH	Control 5HIAA	Apparent 5HIAA from human urine
1.0 (0.1 M HCl)	0.29	0.29
4.0 (0.5 " formate)	0.35	0.32
4.5 (0.5 " citrate)	0.23	0.19
5.5 (0.5 " ")	0.41	0.39
6.0 (0.5 " phosphate)	0.85	0.88
7.0 (0.5 " ")	1.00	1.00

little, if any, material which differs in solubility characteristics from pure 5HIAA.

Dog, rabbit, and rat urine contain ether-extractable acid material which yields the characteristic 5HIAA color with nitrosonaphthol. The material obtained from dog urine was found to be indistinguishable from 5HIAA when subjected to chromatography on paper as described above.

Determination of 5HIAA in Urine—The procedure involves preliminary treatment of the urine with dinitrophenylhydrazine to react with any keto acids present, since these substances when extracted through the procedure interfere with the final color. Normally the interference is slight, but in certain metabolic disorders when large amounts of keto acids are excreted their removal is essential. Extraction with $CHCl_3$ then removes indoleacetic acid, which gives a slight color with nitrous acid. The low distribution coefficient of 5HIAA between ether and water makes it necessary to saturate the aqueous phase with NaCl to extract it into ether. The

5HIAA is returned to buffer of pH 7.0 for colorimetric assay. At pH values above this, the compound becomes progressively more unstable.

To 6 ml. of urine in a 50 ml. glass-stoppered bottle are added 6 ml. of 2,4-dinitrophenylhydrazine reagent.[1] After 30 minutes, 25 ml. of $CHCl_3$

TABLE II

Recovery of 5HIAA Added to Urine

6 ml. samples of urine were taken for analysis.

5HIAA added	Endogenous 5HIAA	Total 5HIAA, calculated	Total 5HIAA, found	Recovery
μmole	*μmole*	*μmole*	*μmole*	*per cent*
0.10	0.10	0.20	0.18	90
	0.10	0.20	0.18	90
	0.14	0.24	0.21	87
	0.12	0.22	0.20	91
	0.06	0.16	0.15	93
	0.05	0.15	0.13	86
	0.06	0.16	0.14	88
	0.19	0.29	0.26	90
	0.15	0.25	0.22	88
	0.14	0.24	0.20	83
	0.05	0.15	0.14	93
	0.16	0.26	0.24	92
	0.54	0.64	0.59	92
0.20	0.10	0.30	0.26	87
	0.22	0.42	0.34	81
	0.14	0.34	0.28	83
	0.16	0.36	0.29	81
0.30	0.10	0.40	0.31	78
	0.05	0.35	0.28	80
	0.10	0.40	0.31	78
	0.08	0.38	0.34	88

are added, and the bottle is shaken for a few minutes and then centrifuged. The organic layer is removed, replaced with a fresh 25 ml. portion of $CHCl_3$, and the extraction repeated. After centrifuging, a 10 ml. aliquot of the aqueous layer is transferred to a 40 ml. glass-stoppered centrifuge tube containing about 4 gm. of NaCl and 25 ml. of ether. The tube is shaken for 5 minutes. Following centrifugation a 20 ml. aliquot of the ether is transferred to another 40 ml. glass-stoppered centrifuge tube containing 1.5 ml. of buffer at pH 7.0. The tube is shaken for 5 minutes, centrifuged, and the ether layer is removed by aspiration. 1 ml. of the aqueous phase is transferred to a 15 ml. glass-stoppered centrifuge tube containing 0.5 ml.

[1] Some urines yield a large precipitate at this step. This should be removed by centrifugation before continuing the assay.

of nitrosonaphthol reagent. Following this, 0.5 ml. of nitrous acid reagent is added, and the sample is then mixed well and warmed at 37° for 5 minutes. 5 ml. of ethyl acetate are then added and the tube is shaken. After separation of the phases and removal of the ethyl acetate by aspiration, a second 5 ml. portion of ethyl acetate is added, and this step is repeated. The final aqueous layer is transferred to a micro cuvette, and the optical density is measured at 540 mμ.[2]

Standards are prepared by treating 6 ml. of solution containing 10 to 200 γ of 5HIAA exactly as for the urine samples. The reagent blank used for the blank setting of the instrument is prepared by treating 6 ml. of water in the same manner.

TABLE III

Urinary Excretion of 5-Hydroxyindoleacetic Acid

Mg. of urinary 5HIAA excreted in 24 hours.*

Day	Dog C	Dog K	Rabbit 1	Rabbit 2	Patient K	Patient W	Patient J	Patient E	Patient C†	Patient MC†
1	2.1	1.7	0.65	0.41	2.6	7.8	8.2	6.1	142.0	384.0
2	2.1	2.0	0.39	0.53	2.6	6.5		5.7		356.0
3	1.5	1.4		0.28	2.0	7.8				321.0
4	2.7	2.0		0.60	2.6	6.7				323.0
5	1.8	1.5			2.5	4.4				332.0
6	1.4	1.8			2.7	6.7				
7	1.4				2.0					

* The animals were normal laboratory animals on a routine diet. The patients were general hospital patients on a normal diet.

† Diagnosed as having intestinal carcinoid.

Table II contains a summary of recoveries of known amounts of 5HIAA added to urine. These average about 85 per cent of the control standards, and urinary values are corrected for this somewhat low recovery. This small loss from urine is due, in part, to the salting out of 5HIAA by the urinary solutes into the $CHCl_3$ used to remove indoleacetic acid.

Specificity—The experimental data presented above for identification of 5HIAA establish the specificity of the assay procedure, since they make use of the same extraction processes and color reaction. Except for the occurrence of trace amounts of a second acidic 5-hydroxyindole substance, detected by paper chromatography, extracts of urine prepared as described in the assay procedure contain no other nitrosonaphthol-reacting material.

Urinary Excretion of 5HIAA—The amounts of 5HIAA excreted daily

[2] The micro cell adapter of the Beckman spectrophotometer has been used for these measurements.

in the urine of the rabbit, dog, and man are reported in Table III. The excretion is fairly constant from day to day in any one individual. However, the variation among normal individuals may be considerable (2 to 8 mg. per day). Of special interest is the relatively huge amount of 5HIAA excreted in the urine of patients with malignant carcinoid, 150 to 400 mg. per day, undoubtedly due to the secretion of large amounts of 5-hydroxytryptamine from the tumor (4) and its subsequent metabolism.

DISCUSSION

Tryptophan is the dietary precursor of 5-hydroxytryptamine (5) and therefore of 5HIAA. The finding of mg. quantities of 5HIAA in urine indicates that an appreciable proportion of tryptophan is metabolized via the 5-hydroxyindole route. These findings are corroborated by the reports of Erspamer (6).

It has been reported previously that 5-hydroxytryptophan, 5-hydroxytryptamine, and 5HIAA when administered to animals are excreted in the urine as 5HIAA (1). It is probable that the metabolism proceeds in this order and that 5HIAA is derived solely from the corresponding amine. However, it is possible that some 5HIAA may be derived from decarboxylation of 5-hydroxyindolepyruvic acid which may arise by transamination of 5-hydroxytryptophan. This possibility is now under investigation.

If the bulk of urinary 5HIAA is derived from 5-hydroxytryptamine, then the amounts of this acid excreted reflect the secretion of the amine. As yet, little is known about the normal functions of 5-hydroxytryptamine or of its implications in pathologic states. Studies on urinary 5HIAA may throw some light on these subjects. The large amounts of 5HIAA excreted in urine of patients with malignant carcinoid corroborate the clinical reports of vasomotor disturbances in this disease due to 5-hydroxytryptamine (4) and the finding of large amounts of 5-hydroxytryptamine in the tumor itself (7). The diagnostic value of urinary 5HIAA assays in suspected malignant carcinoid is obvious.

5HIAA has recently been found in the plant, *Piptadenia peregrina*, which produces large amounts of *N*,*N*-dimethyl-5-hydroxytryptamine (8) and other 5-hydroxyindoleamines.[3] This substance may be present in plants merely as an end-product of metabolism of 5-hydroxyindoleamines. However, it is conceivable that 5-hydroxyindoleacetic acid may have hormonal activities like its unhydroxylated analogue, indoleacetic acid.

SUMMARY

5-Hydroxyindoleacetic acid (5HIAA), a metabolite of 5-hydroxytryptamine, has been identified in the urine of man and a number of laboratory

[3] E. C. Horning, personal communication.

animals. Details of a chemical method for the assay of this metabolite are presented. The normal excretion of 5HIAA in human urine ranges from 2 to 8 mg. per day; patients with argentaffinoma (malignant carcinoid) excrete as much as 350 mg. daily.

We wish to thank Colonel T. W. Mattingly and Dr. John J. Sampson for making available to us the carcinoid urines.

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COMPETITIVE INHIBITION OF 3,4-DIHYDROXYPHENYLALANINE (DOPA) DECARBOXYLASE IN VITRO*

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Studies on the occurrence, properties, and purification of mammalian 3,4-dihydroxyphenylalanine (dopa) decarboxylase (18) have led to a method of preparation of a lyophilized kidney extract which is stable for several months (19). The optimal concentrations of substrate and cofactor have been previously studied in this laboratory in view of the inactivation of the cofactor by chemical reaction with the substrate (20). The substrate specificity has been confirmed and extended (Tables I and II).

Accumulating data on the metabolism of tyrosine, phenylalanine, and their derivatives (10) indicate that dopa or 3,4-dihydroxyphenylserine may be intermediary in the formation of adrenaline and noradrenaline. A few investigators have sought inhibitors for the enzyme dopa decarboxylase (12–14, 21), with the aim of preventing the formation of these two hormones *in vivo*.

It was the purpose of this investigation to study the action of certain substituted cinnamic acids and related substances as inhibitors of the dopa decarboxylase.

Methods

Hog kidney cortex, which has been shown to contain a high dopa decarboxylase activity (9), was used throughout the present investigation for the preparation of enzyme. The enzyme was prepared by the method of Schales and Schales (19), except that the solid material was removed by centrifuging in a refrigerated Servall centrifuge (S-1)[1] at 19,000 × *g* for 30 minutes at 4°. The tan powder resulting from lyophilization was stored at −20°. In 4 months the activity of the enzyme decreased to half its original value. The temperature and length of storage of the fresh hog

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[1] The numbers in parentheses preceded by S refer to the source list.

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kidney influenced the activity of the final powder; the fresh kidney could be stored for 30 days at −20°, 7 days at 0°, or 0.3 day at 25° without reducing the activity of the enzyme preparation appreciably.

The enzyme preparation was tested for the presence of esterase. The spectra of two compounds (Nos. 18, 20, Table IV), which differed only in that Compound 20 is the triacetate of Compound 18, were measured from 320 to 380 mμ. The compounds (0.10 μmole in 4.5 ml. or 2.2×10^{-5} M) were then shaken with 30 mg. of the enzyme preparation in buffer solution (pH 6.8) for 7 minutes at 37° under nitrogen, the enzyme was precipitated with acetone, and the spectra of the filtrates were measured from 320 to 380 mμ with appropriate blanks. It was found that Compound 20 was hydrolyzed in this short time. Furthermore, the hydrolysis of Compound 20 by the enzyme preparation was confirmed by titration of such reaction mixtures with NaOH. This hydrolysis was not affected by addition of sodium fluoride to the reaction mixture. A similar analysis showed that ethyl acetate was hydrolyzed by the enzyme preparation, but this hydrolysis was completely inhibited by sodium fluoride.

The rate of decarboxylation of dopa was measured by the rate of evolution of CO_2 in the conventional Warburg apparatus with an "isooctane" (2,2,4-trimethylpentane) manometer fluid. The powdered kidney extract was dissolved in distilled water and an aliquot containing 15 mg. was pipetted into the main compartment of a conical Warburg vessel with two side arms. The main compartment also contained 2 ml. of 0.1 M phosphate buffer at pH 6.8, 40 γ of calcium pyridoxal-5-phosphate (60 to 70 per cent pure) (S-12), and distilled water to make a total volume of 4.5 ml. One side arm contained 1.98 mg. of L-dopa (10 μmoles) (S-4, S-7) and 0.01 to 10 μmoles of neutralized inhibitor (0.001:1 relative molarity (RM) with respect to the concentration of dopa). The other side arm contained 0.2 ml. of 3 N H_2SO_4 which was tipped into the main compartment at the end of the experiment to liberate the CO_2 trapped in the buffered solution. The temperature was maintained at 37° and the vessels were deaerated with nitrogen. After removal of the air, the vessels were closed and equilibrated for 10 minutes. The substrate and the addition substance were tipped into the vessel on a flashing signal from an interval timer. A reading was made after 5 minutes, the 3 N H_2SO_4 was tipped into the flask 2 minutes later, and a final reading was taken after 15 minutes of equilibration. The average Q_{CO_2} value obtained was 57 μl. per mg. per hour, and the decarboxylation of L-dopa was usually 50 per cent complete in the 7 minutes.

The substrate studies were carried out in the same manner, except that one side arm contained 10 μmoles of compound instead of L-dopa plus inhibitor.

Tyrosine decarboxylase was prepared by the method of Gale (6) from *Streptococcus faecalis* 6783 (S-3). Tyrosinase prepared from mammalian melanoma was obtained from Dr. A. B. Lerner (S-4). Glutamic acid decarboxylase was prepared from *Clostridium welchii* 6784 (S-5), according to the method of Meister, Sober, and Tice (15). Histidine decarboxylase was prepared from *Escherichia coli* (S-5) according to the procedure of Nash (16). Succinic acid dehydrogenase was prepared from beef heart by the method of Weil-Malherbe (24).

The rat liver slices used in Table X were prepared on ice by the razor blade-glass slide method of slicing. The translucent slices were stored in iced Ringer-phosphate solution until sufficient tissue had been sliced to allow seven slices (average total, 500 mg. wet weight) of liver per vessel. The main compartment of the Warburg vessels contained the slices and 3.0 ml. of Ringer-phosphate solution at pH 7.4. The side arms contained 10 μmoles of inhibitor. The center wells contained a paper wick and 0.3 ml. of 10 per cent NaOH. The flasks were equilibrated with air at 37° and the stop-cocks were closed. The endogenous oxygen uptake was followed for 15 minutes; then the compounds were tipped in from the side arms and the uptake was followed for an additional hour.

For the chromatographic studies[2] larger volumes of substrate and inhibitor were incubated with proportional amounts of enzyme in the Dubnoff incubator (S-2) for 30 minutes. Four flasks run as controls contained 10 mg. of dopa, 150 mg. of enzyme, 2 ml. of 0.2 M phosphate buffer at pH 6.8, 50 γ of calcium pyridoxal-5-phosphate, and water to a total volume of 21.5 ml. 50 mg. of ascorbic acid were added to each batch to prevent oxidation of dopa and the resulting 3,4-dihydroxyphenethylamine (dopamine). Four flasks run for inhibition study contained all the above constituents plus 4 mg. of caffeic acid (0.44 RM). After 30 minutes incubation at 37° under nitrogen, the control flasks were pooled and the protein was precipitated with an equal volume of 10 per cent trichloroacetic acid. The solution was filtered and the filtrate was extracted four times with equal volumes of ether to remove the trichloroacetic acid. The pooled solution containing the inhibitor was treated in the identical manner. The extracted solutions were warmed under vacuum to remove the ether and then were lyophilized. The residues were taken up in minimal amounts of 0.01 N HCl, spotted on Whatman No. 1 paper, and developed (descending) with isopropyl alcohol, glacial acetic acid, and water (70:5:25)[3] and the paper was sprayed with the potassium ferricyanide-ferric sulfate reagent of Goldenberg *et al.* (7).

[2] We wish to thank Dr. William Drell for carrying out the paper chromatography, and also for valuable discussion and criticism of this manuscript.

[3] Drell, W., unpublished.

RESULTS AND DISCUSSION

Some characteristics of the dopa decarboxylase preparation were studied. Its activity was completely inhibited by simultaneous addition of substrate and sufficient 4-chloromercuribenzoic acid (S-8) to give a concentration of 10^{-4} M (0.043 RM), but was not affected if the enzyme was protected first with glutathione (2 RM). Oxophenarsine hydrochloride (10^{-4} M or 0.043 RM) also inhibited 74 per cent of the enzyme activity, but only if equilibrated with the enzyme before the substrate was added, and this inhibition was similarly prevented by glutathione. This indicates that the enzyme contains an essential thiol group, but one which is not readily attacked.

The dialyzed enzyme was not reactivated either by pyridoxal-5-phosphate or by phosphate alone, but 95 per cent of the activity[4] was regained when both pyridoxal-5-phosphate and phosphate were added. Arsenate was as effective as phosphate, pyrophosphate was only slightly active, and arsenite, iodide, fluoride, and sulfate were ineffective. Adenosine-5-phosphate, adenosine diphosphate, adenosine triphosphate, cocarboxylase, coenzyme I, and cytochrome *c* did not increase the activity of undialyzed enzyme, and 2,4-dinitrophenol did not inhibit the activity appreciably.

The inactivity of metal-chelating agents (8-hydroxyquinoline, 1-(*o*-hydroxyphenyl)isoquinoline (S-9), disalicylalethylenediamine (S-9), 2,3-dimercaptopropanol (S-10), and ethylenediaminetetraacetic acid) and the complete reactivation of the dialyzed enzyme without addition of metal indicate that the enzyme is not metal-dependent or that the metal is tightly bound.

The decarboxylations by dopa decarboxylase of 3,4-dihydroxyphenylalanine (18), 2-hydroxyphenylalanine (2), 3-hydroxyphenylalanine (3), 2,3-dihydroxyphenylalanine (4, 22), 2,4-dihydroxyphenylalanine (23), 2,5-dihydroxyphenylalanine (3), 2,6-dihydroxyphenylalanine (22), 3,5-dihydroxyphenylalanine (23), and *erythro*-3,4-dihydroxyphenylserine (5) have been reported. Most of these decarboxylations have been repeated in this laboratory (Table I). Moreover, *erythro*-3-hydroxyphenylserine, 2-methyl-3,4-dihydroxyphenylalanine, and 6-methyl-3,4-dihydroxyphenylalanine were substrates, although poor ones, and 5-methyl-3,4-dihydroxyphenylalanine was not. The compounds listed in Table II are neither substrates nor inhibitors of the enzyme.

Of all the compounds with a phenylalanine structure tested under our conditions, only two, α-methyl-β-(3-hydroxy-4-methoxyphenyl)alanine and α-methyl-3,4-dopa (Table VI, Compounds 17, 18), were found to have

[4] The activity was compared to that of an undialyzed control sample which had been standing for the same length of time (16 hours) at the same temperature (4°); the control had 90 per cent of its original activity.

any inhibitory action. These results confirm those of Sourkes (21); the variations are due to differences in the method of assay.

TABLE I

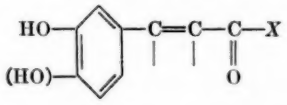
Substrates of Dopa Decarboxylase

Compound No.	Position No.						Source No.	CO_2
	2	3	4	5	6	β		
								μl.
1	OH	H	H	H	H	H	14	96
2	H	OH	"	"	"	"	15	83
3	OH	"	"	"	"	"	11	118
4	"	H	OH	"	"	"	12	53
5	"	"	H	OH	"	"	12, 13	72
6	H	OH	OH	H	"	"	4, 7	91
7	CH_3	"	"	"	"	"	11	17
8	H	"	"	"	CH_3	"	11	42
9	"	"	H	"	H	OH	16 (Erythro)	25*
10	"	"	OH	"	"	"	16 "	60*

Each flask contained 10 μmoles of substrate in one side arm, 0.2 ml. of 3 N H_2SO_4 in the other side arm, 15 mg. of kidney powder, and 2.0 ml. of 0.1 M phosphate buffer at pH 6.8, 40 γ of calcium pyridoxal-5-phosphate, and water to 4.5 ml. in the main compartment; gas, nitrogen; temperature, 37°; time, 7 minutes.

* Time, 1.5 hours.

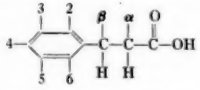
The best inhibitors found in this study have the basic structure



where *X* is OH, O-alkyl, alkyl, or aryl. Compounds with hydroxyl groups on both the 3 and 4 positions are better inhibitors than those with just a 3-hydroxyl group. Thus, 3-hydroxycinnamic acid (*X* is OH) is moderately active as an inhibitor, a concentration of 4.6×10^{-4} M or 0.2 RM producing 37 per cent inhibition in 7 minutes upon simultaneous addition of inhibitor and substrate. Caffeic acid (3,4-dihydroxycinnamic acid; *X* is OH) is more active, 0.2 RM producing 74 per cent inhibition. The kind of inhibition (competitive or non-competitive) was investigated by the method of Lineweaver and Burk (11). A plot of the data (Fig. 1) showed

TABLE II

Non-Substrates of Dopa Decarboxylase



Compound No.	Position No. 2	3	4	5	β	α	Source No.
1	H	H	H	H	H	NH_2	6
2	"	CH_3	"	"	"	"	17
3	"	H	F	"	"	"	18
4	"	"	NO_2	"	"	"	17
5	Cl	"	Cl	"	"	"	17
6	H	Cl	"	"	"	"	17
7	"	H	NH_2	"	"	"	17
8	"	"	CH_3O	"	"	"	18
9	C_2H_5O	"	H	NO_2	"	"	17
10	OH	"	"	"	"	"	17
11	H	"	OH	H	"	"	6
12	"	CH_3	"	"	"	"	18
13	"	NO_2	"	"	"	"	6
14	"	F	"	"	"	"	19, 20
15	"	NH_2	"	"	"	"	6
16	"	I	"	I	"	"	6
17	"	OH	"	CH_3	"	"	11
18	"	"	"	H	"	$NHCH_3$	21
19	"	H	"	"	"	NHCHO	4
20	"	"	"	"	"	$NHCOCH_3$	4
21	"	CH_3O	CH_3O	"	"	$NHCOC_6H_5$	12
22	"	H	H	"	C_6H_5	NH_2	17
23	"	"	"	"	OH	" (Threo)	6
24	Cl	"	"	"	"	"	22
25	H	"	Cl	"	"	"	22
26	"	NO_2	H	"	"	" (Erythro)	23
27	"	H	NO_2	"	"	" (Threo)	22, 23
28	"	"	NH_2	"	"	" (Erythro)	23
29	"	OH	H	"	"	" (Threo*)	16
30	"	H	OH	"	"	" "	16
31	"	OH	"	"	"	" "	16
32	"	"	H	"	NH_2	H	24
33	2-(3-Hydroxyphenyl)glycine						25
34	2-(4-Hydroxyphenyl)glycine						27
35	2-(3,4-Dihydroxyphenyl)glycine						26
36	2-(3-Methoxy-4-hydroxyphenyl)glycine						26
37	*o*-Benzenedialanine						17
38	Thyroxine						6

TABLE II—*Concluded*

Compound No.	Position No.						Source No.
	2	3	4	5	β	α	
39	1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid						17
40	Histidine						6
41	5-Hydroxytryptophan						28
42	Leucenol						31
43	3-(2-Thienyl)serine						29
44	3-(2-Furyl)serine						32
45	α-Acetamido-3-hydroxycinnamic acid						15
46	4-Hydroxyphenylpyruvic acid						18, 30

Conditions same as those in Table I.
* Time, 1.5 hours.

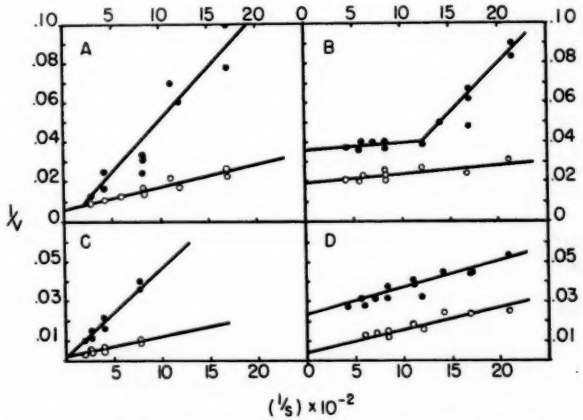
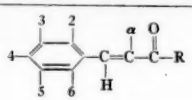


FIG. 1. Inhibition of dopa decarboxylase; Lineweaver-Burk analysis. The lower curve in each section represents the decarboxylation of dopa in the absence of any supplement; the upper curve represents the decarboxylation of dopa in the presence of the following compounds: *A*, 5-(3,4-dihydroxycinnamoyl)salicylic acid; *B*, 3,5-diiodosalicylic acid; *C*, caffeic acid; *D*, 4-hydroxyphenylpyruvic acid. Different batches of enzyme were used with the various compounds. The concentration of 4-hydroxyphenylpyruvic acid was 1.1×10^{-3} M, that of caffeic acid and 3,5-diiodosalicylic acid 4.5×10^{-4} M, and that of 5-(3,4-dihydroxycinnamoyl)salicylic acid 1.1×10^{-6} M (1.0 RM cysteine was added in this case). v = microliters of CO_2 produced in 7 minutes; s = molar concentration of dopa.

TABLE III

Inhibition by Cinnamic Acids and Derivatives



Compound No.	Position No.							Source No.	Relative molarity	Inhibition
	2	3	4	5	6	α	R			per cent
1	H	H	H	H	H	H	OH	6	1.0	0
2	OH	"	"	"	"	"	"	33	1.0	52
3	"	Br	"	Br	"	"	"	34	0.2	65
4	H	OH	"	H	"	"	"	35	0.2	37
5	"	"	"	"	"	CH_3	"	36	0.2	31
6	"	"	"	"	"	C_2H_5	"	36	0.2	27
7	"	"	"	"	"	$NHCOCH_3$	"	15	0.2	7
8	"	"	"	"	SO_3H	H	"	24	0.2	31
9	"	H	OH	"	H	"	"	37	0.2	7
10	CH_3O	CH_3O	H	"	"	"	"	38	1.0	8
11	OH	H	OH	"	"	"	"	24	1.0	9
12	"	"	H	"	OH	"	"	31	0.1	84
13	H	C_2H_5O	C_2H_5O	"	H	"	"	38	1.0	5
14	"	OH	CH_3O	"	"	"	"	39	1.0	19
15	"	CH_3O	OH	"	"	"	"	38	1.0	5
16	"	C_2H_5O	"	"	"	"	"	38	1.0	0
17	"	OH	"	"	"	"	"	38	0.2	74
18	"	CH_3COO	CH_3COO	"	"	"	"	24	0.2	60
19	"	SH	H	"	"	"	"	24	0.05	78
20	Cl	H	"	"	"	"	"	40	1.0	36
21	H	"	Cl	"	"	"	"	40	1.0	8
22	Cl	"	"	"	"	"	"	40	1.0	41
23	H	"	CN	"	"	"	"	38	1.0	17
24	"	NH_2	H	"	"	"	"	24	1.0	10
25	"	NO_2	"	"	"	"	"	6	1.0	15
26	"	"	"	"	"	C_2H_5	"	36	1.0	0
27	"	OH	"	"	"	H	OCH_3	41	0.2	60
28	"	"	CH_3O	"	"	"	"	39, 42	1.0	37
29	"	"	OH	"	"	"	OC_2H_5	6	0.15	90
30	"	"	"	"	"	"	O-Quinic acid	43	0.1	70
31	"	CH_3COO	CH_3COO	"	"	"	$OCH_2CH_2N(CH_3)_2$	24	0.2	55
32	Cl	H	H	"	"	"	"	40	1.0	17
33	H	OH	"	"	"	"	NH_2	44	0.2	19
34	"	"	"	"	"	"	$NHCH_2COOH$	24	0.2	24
35	OH	H	"	"	"	"	CH_3	6	0.1	31
36	H	OH	"	"	"	"	"	24	0.1	30

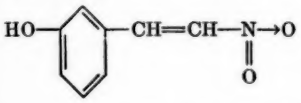
Conditions the same as those in Table I, except that the indicated amount of inhibitor neutralized with 0.1 N NaOH was combined with the 10 μmoles of substrate (L-dopa) in one side arm. Compounds 27, 28, 29, 33, 34, 35, and 36 were dissolved in 0.2 ml. of propylene glycol.

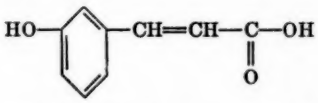
that the inhibition by caffeic acid was competitive. Similar results (not shown) were obtained with 3-hydroxycinnamic acid.

Substitution of a 3-mercapto group for the 3-hydroxyl group increases the activity even more, since 0.05 RM causes 78 per cent inhibition. Other mercapto compounds with variations in *X* are under investigation.

Substitution in the side chain of the above structure, as in α-methyl- and α-ethyl-3-hydroxycinnamic acids (Table III, Compounds 5, 6), does not change the activity from that of the unbranched compound.

The compound 3-hydroxy-ω-nitrostyrene (Table VII, Compound 22), which is structurally similar to 3-hydroxycinnamic acid, is a moderately active inhibitor.

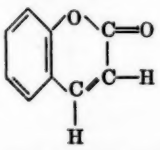




Esters of cinnamic acids (*X* is O-alkyl) are generally as good as or better inhibitors than the acids themselves; thus, methyl 3-hydroxycinnamate (Table III, Compound 27) is twice as active as the free acid. Amides (*X* is NH_2; Table III, Compounds 33, 34) are less active than the free acid.

The use of an alkyl group for *X*, as in 3-hydroxybenzalacetone (*X* is CH_3; Table III, Compound 36), results in a better inhibitor than 3-hydroxycinnamic acid, and an aryl group, as in 3-hydroxychalcone (*X* is C_6H_5), is still better, 0.01 and 0.10 RM producing 24 and 100 per cent inhibition, respectively. Substitution of heterocyclic rings, such as furan and thiophene (Table IV, Compounds 21, 22), for *X* gives compounds not as effective as the chalcone, but these are still better than the groups previously discussed.

When hydroxyl groups are present in only the 2 or 4 position of the ring, the activity of the compounds decreases, those with a 2-hydroxyl group being somewhat more active than those with a 4-hydroxyl group. Again, the activity is increased as *X* is changed from OH to alkyl to aryl. Coumarins (Table IX, Compounds 15, 16, 17, 18, 19), which may be considered as inner esters of 2-hydroxycinnamic acid,

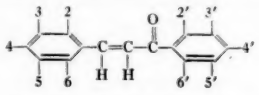


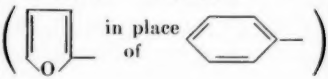
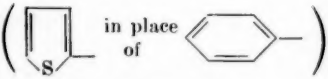
were completely inactive or only slightly active.

A class of heterocyclic compounds called flavones, which may be considered derivatives of the open chain chalcones, has been investigated.

TABLE IV

Inhibition by Chalcones and Derivatives



Compound No.	Position No. 2	3	4	5	2′	3′	4′	5′	Source No.	Relative molarity	Inhibition per cent
1	H	H	H	H	OH	OH	OH	H	9	1.0	24
2	OH	"	"	"	H	H	CN	"	38	0.2	0
3	"	"	"	"	OH	"	H	OH	9	0.1	35
4	"	Br	"	Br	H	COOH	OH	H	24	0.1	65
5	H	OH	"	H	"	H	H	"	9	0.1	100
										0.01	24
6	"	"	"	"	"	"	OCH_2COOH	"	24	0.1	34
7	"	"	"	"	"	COOH	OH	"	24	0.01	85
										0.001	27
8	"	CH_3COO	"	"	"	"	CH_3COO	"	24	0.01	50
9	"	H	OH	"	"	H	OH	"	9	0.2	16
10	OH	"	"	"	"	COOH	"	"	24	0.1	10
11	H	CH_3O	"	"	OH	H	H	"	9	1.0	15
12	"	OH	"	"	H	"	"	"	9	0.1	100
										0.05	85
13	"	"	"	"	OH	"	"	"	9	0.1	90
14	"	"	"	"	"	"	"	H*	9	0.1	65
15	"	"	"	"	"	"	OH	H	9	0.1	100
										0.05	85
16	"	"	"	"	"	"	"	H†	45	0.1	82
17	"	"	"	"	"	"	CH_3COO	H	9	0.1	75
18	"	"	"	"	H	COOH	OH	"	24	0.001	87
19	"	"	"	"	"	$COOC_2H_5$	"	"	24	0.01	30
20	"	CH_3COO	CH_3COO	"	"	COOH	CH_3COO	"	24	0.001	64
21	"	OH	H	"					24	0.1	50
22	"	"	"	"					24	0.1	50

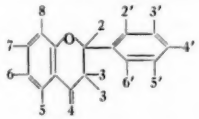
Conditions the same as those in Table III, but all compounds were dissolved in 0.2 ml. of propylene glycol except Compound 19 which was dissolved in 0.2 ml. of polyethylene glycol and Compounds 4, 7, 8, 18, and 20 which were dissolved in water.

* x-Sulfonic acid.

† x-Glycoside (coreopsin).

TABLE V

Inhibition by Flavones and Derivatives

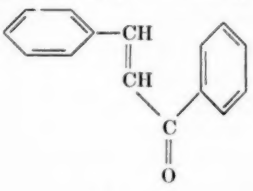


Compound No.	Position No.										Source No.	Relative molarity	Inhibition
	2	3	3	4	5	7	8	3′	4′	6′			
													per cent
1	*	*	H	O	H	H	H	H	H	H	9	0.2	0
2	*	*	"	"	"	"	"	OH	OH	"	9	0.1	90
												0.01	59
3	*	*	OH	"	OH	OH	"	"	"	SO_3H	9	0.05	55
4	*	*	"	"	"	"	O (Glucose)	"	"	H	46	0.1	77
5	*	*	O (Rutinose)	"	"	"	H	"	"	"	47	0.1	5
6	H	H	H	"	"	"	"	"	"	"	9, 47	0.1	86
7	"	"	"	"	"	"	"	"	OCH_3	"	39	1.0	86
8	"	"	"	"	"	"	"	OCH_3	OH	"	9, 47	1.0	61
9	"	"	OH	H_2	"	"	"	OH	"	"	47	0.1	50

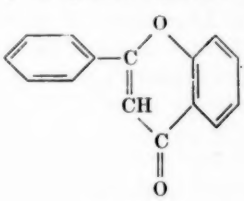
Conditions the same as those in Table III, except that all compounds were dissolved in 0.2 ml. of propylene glycol.

* Double bond in the 2,3 position.

Again, compounds with hydroxyls on the 3 and 4 positions of the left-hand ring of the flavone structure presented[5] are very active inhibitors (Table V, Compounds 2, 3, 4). Rutin (Table V, Compound 5), however, though



Chalcone



Flavone

very similar to gossypin (Table V, Compound 4), has its glycoside group in

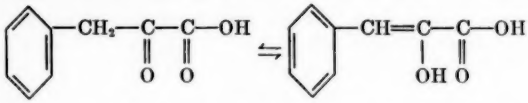
[5] This formula is the mirror image of that conventionally used (Table V), but is written in this manner to show the relationship to the chalcone formula. The numbering system is shown in Table V.

a position that must interfere sterically with attachment to the enzyme. Catechin (Table V, Compound 9) is of interest, since it is quite active even though it does not have a carbonyl group.

Acetyl derivatives of several of the most active inhibitors (Table III, Compounds 18, 31; Table IV, Compounds 8, 17, 20) were prepared for testing *in vivo* (17). Since the enzyme preparation contains an esterase (see above), it was expected that these acetates would be only slightly less active than their parent compounds. This was found to be the case. The inhibition of the dopa decarboxylase by 5-(3,4-diacetoxycinnamoyl)acetylsalicylic acid (Table IV, Compound 20) was not affected by the addition of sodium fluoride, hexaethyl tetraphosphate, or diisopropyl fluorophosphate.

The change in inhibitory action produced by removal of the double bond in the side chain of the basic structure (shown above) is variable. Thus, 3-hydroxyhydrocinnamic acid (Table VI, Compound 2) is inactive, while 3,4-dihydroxyhydrocinnamic acid (Table VI, Compound 4) is only slightly less active than caffeic acid. Flavanones, which do not have the double bond in the heterocyclic ring characteristic of flavones, are still active (Table V, Compounds 6, 7, 8).

Phenylpyruvic acids, which have a different side chain than the basic structure, are still active, possibly because of keto-enol tautomerism (shown in the accompanying structures). Pyruvic acid itself (lithium salt



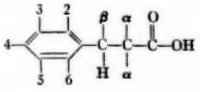
(S-8)) is inactive. The unsubstituted phenylpyruvic acid (Table VI, Compound 22) is moderately active, and the activity improves when hydroxyl groups are substituted in the 3 position (Table VI, Compound 23) and in the 3,4 positions (Table VI, Compound 26). Phenylthiopyruvic acid (Table VI, Compound 28) is as active as the oxygen analogue. Phenyllactic acid (Table VI, Compound 13) has no activity.

An inhibitory effect of 4-hydroxyphenylpyruvic acid on dopa decarboxylase (40 per cent at 1.3 RM) has been reported by Martin, Brendel, and Beiler (14). This compound has been investigated in our laboratory and treatment of the data by the method of Lineweaver and Burk (11) indicates that the inhibition is non-competitive (Fig. 1). The effect of 4-hydroxyphenylpyruvic acid (Table VI, Compound 24) is no greater than that of phenylpyruvic acid (Table VI, Compound 22).

Reducing the side chain of the basic structure from 3 to 2 carbon atoms removed most of the activity. Thus, 3-hydroxyphenylacetic acid (Table VII, Compound 2) produces 11 per cent inhibition at 1.0 RM, and 3,4-

TABLE VI

Inhibition by Hydrocinnamic Acids and Derivatives



Compound No.	Position No.								Source No.	Relative molarity	Inhibition
	2	3	4	5	6	β	α	α			
											per cent
1	H	H	H	H	H	H	H	H	6	1.0	0
2	"	OH	"	"	"	"	"	"	48	1.0	0
3	"	H	OH	"	"	"	"	"	49	1.0	9
4	"	OH	"	"	"	"	"	"	50	0.22	63
5	Cl	H	Cl	"	"	"	"	"	40	1.0	38
6	I	OH	I	"	I	"	"	"	36	0.1	90
7	"	"	"	"	"	"	"	CH_3	36	0.2	91
8	"	"	"	"	"	"	"	C_2H_5	36	0.2	71
9	OH	I	H	I	H	"	"	C_6H_5	36	0.2	100
10	I	OH	I	H	"	"	"	"	36	0.2	78
11	H	"	"	"	I	"	"	C_2H_5	36	0.2	69
12	I	NH_2	"	"	"	"	"	"	51	0.1	22
13	H	H	H	"	H	"	OH	H	52	1.0	0
14	"	"	OH	"	"	"	"	"	70	1.0	24
15	"	CH_3O	H	"	"	"	NH_2	CH_3	12	1.0	13
16	"	"	CH_3O	"	"	"	"	"	12	1.0	0
17	"	OH	"	"	"	"	"	"	12	1.0	49
18	"	"	OH	"	"	"	"	"	12	0.1	37
19	"	CH_3O	CH_3O	"	"	"	$NHCOC_6H_5$	H	12	1.0	0
20	"	OH	H	"	"	NH_2	H	"	24	1.0	0
21	OH	Br	"	Br	"	SO_3H	"	"	34	0.2	39
22	H	H	"	H	"	H	—O—*		52	1.0	60
23	"	OH	"	"	"	"	—O—		53	0.5	72
24	"	H	OH	"	"	"	"		18, 30	1.0	60
25	OH	"	H	OH	"	"	"		54	1.0	60
26	H	OH	OH	H	"	"	"		13	0.1	60
27	NO_2	H	H	"	"	"	"		6	1.0	58
28	H	"	"	"	"	"	—S—		55	1.0	67

Conditions the same as those in Table III.

* This compound is phenylpyruvic acid.

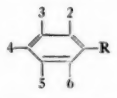
dihydroxyphenylacetic acid (Table VII, Compound 6) produces 88 per cent inhibition at 1.0 RM. Substituted 2-phenylglycines, mandelic acids, and acetophenones (Table VII) are also inactive.

Most compounds with a 1-carbon side chain (benzoic acid unsubstituted

or substituted in the 2, 3, or 4 position with chlorine, bromine, iodine, nitro, or amino, in the 2 or 4 position with fluorine or carboxyl, in the 2 or 3 position with hydroxyl or methoxyl, and in the 4 position with methyl)

TABLE VII

Inhibition by Compounds with 2-Carbon Side Chains

Compound No.	 Position No. 2	3	4	5	R	Source No.	Relative molarity	Inhibition
								per cent
1	H	H	H	H	CH_2—COOH	6	1.0	0
2	"	OH	"	"	"	36	1.0	11
3	"	F	OH	"	"	20	1.0	0
4	OH	H	H	OH	"	37	1.0	28
5	CH_3O	"	"	CH_3O	"	37	1.0	16
6	H	OH	OH	H	"	56	1.0	88
7	"	CH_3O	CH_3O	"	"	6	1.0	18
8	"	H	H	"	CHOH—COOH	6	1.0	0
9	"	OH	OH	"	CHOH—$COOC_2H_5$	38	1.0	0
10	"	—O—CH_2—O—		"	CHOH—COOH	38	1.0	0
11	"	H	H	"	$CHNH_2$—COOH	6	1.0	0
12	"	OH	"	"	"	25	1.0	0
13	"	H	OH	"	"	27	1.0	0
14	"	CH_3O	"	"	"	26	1.0	0
15	"	OH	"	"	"	26	1.0	16
16	"	H	H	"	CO—CH_3	6	1.0	0
17	OH	"	"	"	"	57	1.0	0
18	H	OH	"	"	"	58	1.0	17
19	"	NH_2	"	"	"	6	0.2	8
20	"	OH	"	"	O—CH_2—COOH	24	1.0	32
21	"	H	CH_3CO	"	"	59	1.0	0
22	"	OH	H	"	CH=CH—NO_2	60	1.0	73

Conditions the same as those in Table III, except Compounds 9, 16, 17, 18, and 19 dissolved in 0.2 ml. of propylene glycol.

are inactive at 1.0 RM. Salicylic acids are generally inactive, unless substituted with halogens or nitro groups as discussed below. However, 2-mercaptobenzoic acid (Table VIII, Compound 20) produces 50 per cent inhibition at 1.0 RM, and its oxidation product dithiosalicylic acid (Table IX, Compound 25) produces 78 per cent inhibition at 1.0 RM. Hydroxynaphthoic acids (Table IX, Compounds 8, 9, 10) are somewhat more po-

TABLE VIII

Inhibition by Benzoic Acids

Compound No.	Position No.					Source No.	Relative molarity	Inhibition
	2	3	4	5	6			
								per cent
1	OH	H	OH	H	H	6	1.0	0
2	"	"	H	OH	"	6	1.0	21
3	H	OH	OH	H	"	61	1.0	0
4	OH	H	"	"	OH	6	1.0	16
5	H	OH	"	OH	H	6	1.0	42
6	OH	H	NH_2	H	"	6	1.0	0
7	"	"	H	NH_2	"	6	1.0	28
8	NH_2	OH	"	H	"	6	1.0	17
9	OH	NO_2	"	"	"	6	1.0	0
10	"	H	NO_2	"	"	6	1.0	26
11	"	"	H	NO_2	"	6	1.0	0
12	"	NO_2	"	"	"	6	0.1	22
13	"	H	"	Br	"	6	1.0	0
14	"	Br	"	"	"	62	0.1	33
15	H	OH	Br	H	Br	63	1.0	36
16	OH	I	H	I	H	6	0.1	47
17	H	"	OH	"	"	6	1.0	0
18	OH	C_6H_5	H	H	"	6	1.0	77
19	"	H	"	CH_3CO	"	64	1.0	0
20	SH	"	"	H	"	6	1.0	50
21	NH_2	"	NO_2	"	"	6	1.0	0
22	"	"	H	NO_2	"	65	1.0	28
23	"	I	"	I	"	27	0.2	59
24	H	NO_2	"	NO_2	"	6	0.1	10
25	Cl	H	"	"	"	6	1.0	0
26	"	"	Cl	H	"	6	1.0	4
27	"	"	H	Cl	"	40	1.0	0
28	H	Cl	Cl	H	"	6	1.0	17
29	"	Br	H	Br	"	8	0.1	10
30	I	I	"	I	"	6	1.0	83

Conditions the same as those in Table III.

tent than hydroxybenzoic acids. The compounds 3-nitrobenzaldehyde (S-6), 4-nitrobenzaldehyde (S-6), 4-chlorobenzaldehyde (S-6), and 2-hydroxy-5-chlorobenzaldehyde are all inactive.

Phenols (Table IX, Compounds 1 to 7) are generally not active.

Substitution of halogen atoms or nitro groups on the benzene ring usually increases the activity of the inhibitors. For example, 4-chlorocinnamic acid (Table III, Compound 21) and 2-chlorocinnamic acid (Table III, Compound 20) produce 8 and 36 per cent inhibition at 1.0 RM, respectively, 2,4-dichlorocinnamic acid (Table III, Compound 22) produces 41 per cent inhibition at 1.0 RM, and 3-nitrocinnamic acid produces 15 per cent inhibition at the same concentration. Even some halogenated benzoic acids (Table VIII) are moderately active. When halogen atoms or nitro groups are associated with hydroxyl groups on the benzene ring, the activity is much greater. The compound 3,5-dibromo-2-hydroxycinnamic acid (No.

TABLE IX

Inhibition by Miscellaneous Compounds

Compound No.		Source No.	Relative molarity	Inhibition
				per cent
1	Phenol	6	0.4	6
2	Catechol	6	3.0	50
3	Resorcinol	6	0.4	13
4	Hydroquinone	6	0.1	0
5	Phloroglucinol	6	1.0	45
6	4-Chlororesorcinol	66	1.0	58
7	2,4-Dinitrophenol	6	1.0	13
8	1-Hydroxy-2-naphthoic acid	65	1.0	61
9	3-Hydroxy-7-sulfo-2-naphthoic acid	27	1.0	31
10	3,5-Dihydroxy-2-naphthoic acid	65	1.0	83
11	Propiophenone	6	1.0	0
12	Phenylacetone	6	1.0	0
13	Phenylbutyric acid	6	1.0	10
14	Benzalpyruvic "	67	1.0	0
15	Coumarin	6	1.0	0
16	3-Hydroxycoumarin	68	0.1	0
17	3-Methylumbelliferone	66	1.0	33
18	Esculetin-4-carboxylic acid	9	1.0	35
19	Esculin	6	1.0	0
20	Phloretin	9	1.0	97
21	Leptosin	9	0.1	48
22	β-(2-Thienyl)acrylic acid	38	1.0	0
23	3-Hydroxy-2-phenylcinchoninic acid	69	1.0	74
24	Quinic acid	47	1.0	0
25	Dithiosalicylic acid	6	1.0	78
26	1,2-Naphthoquinone-4-sulfonic acid	6	0.1	71

Conditions the same as those in Table III, except Compounds 11, 12, 15, 16, 17, 19, 20, and 21 were dissolved in 0.2 ml. of propylene glycol.

TABLE X

Effect of Cinnamic Acids on Other Enzyme Systems

Enzyme	Substrate	Without supplement	+ 3-Hydroxycinnamic acid			+ Caffeic acid		
			Relative molarity		Inhibition	Relative molarity		Inhibition
		*µl.**		*µl.**	*per cent*		*µl.**	*per cent*
Tyrosine decarboxylase	Dopa, pH 7.5	50				0.5	50	0
	Tyrosine, pH 5.5	120	0.5	125	0	0.5	130	0
	" " 6.8	67	0.5	81	0	0.5	51	24
	" " 7.5	98	0.5	96	0	0.5	100	0
Glutamic acid decarboxylase	Glutamic acid	206†	0.2	203	0	0.2	206	0
Histidine decarboxylase	Histidine	150	0.6	153	0	0.6	147	0
Tyrosinase	Dopa	102	1.0	86	15	1.0	92	10
	Tyrosine	72				1.0	30	58
Succinic acid dehydrogenase	Succinic acid	127				0.5	127	0
	" " + glutathione	206				0.5	207	0
Rat liver slices		1030		1030	0		1200	0

Each flask contained the following enzyme and components: *tyrosine decarboxylase*, 10 mg. of acetone powder of *Streptococcus faecalis*, 16 µmoles of the L form of substrate (0.4 ml. of 0.04 M), 8 µmoles of inhibitor (0.4 ml. of 0.02 M), 2 ml. of 0.3 M buffer (acetate at pH 5.5, phosphate at pH 6.8 and 7.5), 40 γ of calcium pyridoxal-5-phosphate; air; 37°; 30 minutes; *glutamic acid decarboxylase*, 15 mg. (dry weight) of *Clostridium welchii*, 10 µmoles of L-glutamic acid (1.46 mg.), 2 µmoles of inhibitor, 0.3 ml. of 3 N acetate buffer at pH 4.9, 40 γ of calcium pyridoxal-5-phosphate, 0.2 ml. of 3 N H_2SO_4, water to 4.5 ml.; air; 37°; 15 minutes; *histidine decarboxylase*, 15 mg. (dry weight) of *Escherichia coli*, 15 µmoles of L-histidine (0.5 ml. of 0.03 M), 9 µmoles of inhibitor, 2 ml. of 0.05 M phthalate buffer at pH 4.0, 40 γ of calcium pyridoxal-5-phosphate, water to 4.5 ml.; nitrogen; 30°; 1 hour; *tyrosinase*, 0.2 ml. of enzyme solution, 2.8 µmoles of the L form of substrate, 2.8 µmoles of inhibitor, 1.5 ml. of 0.1 M phosphate buffer at pH 6.8, water to 3.3 ml., 10 per cent NaOH in the center well; air; 37°; 2 hours; *succinic acid dehydrogenase*, 50 mg. (dry weight) of enzyme, 20 µmoles of succinic acid, 10 µmoles of inhibitor, 10 µmoles of glutathione, 1.4 ml. of 0.1 M phosphate buffer at pH 6.8, 0.5 ml. of 0.5 per cent brilliant cresyl blue, water to 3.3 ml., 10 per cent NaOH in the center well; air; 37°; 30 minutes; *rat liver slices*, 500 mg. (wet weight) of liver slices (average Q_{O_2} (wet weight) = 2), 10 µmoles of inhibitor, 3.0 ml. of 0.1 M phosphate buffer at pH 7.4, 10 per cent NaOH in the center well; air; 37°; 1 hour.

* Microliters of CO_2 evolved for the first three enzymes; microliters of O_2 absorbed for the last three enzymes.

† Not affected by 5-(3-hydroxycinnamoyl)salicylic acid at 0.2 RM or by 5-(3,4-dihydroxycinnamoyl)salicylic acid at 0.2 RM.

3, Table III) is a good inhibitor (65 per cent inhibition at 0.2 RM), and the corresponding chalcone (Table IV, Compound 4) is even better (65 per cent inhibition at 0.1 RM). Iodination of the inactive 3-hydroxyhydrocinnamic acid (Table VI, Compound 2) results in a powerful inhibitor, 2,4,6-triiodo-3-hydroxyhydrocinnamic acid (Table VI, Compound 6), which produces 90 per cent inhibition at 0.1 RM. Substitution in the side chain

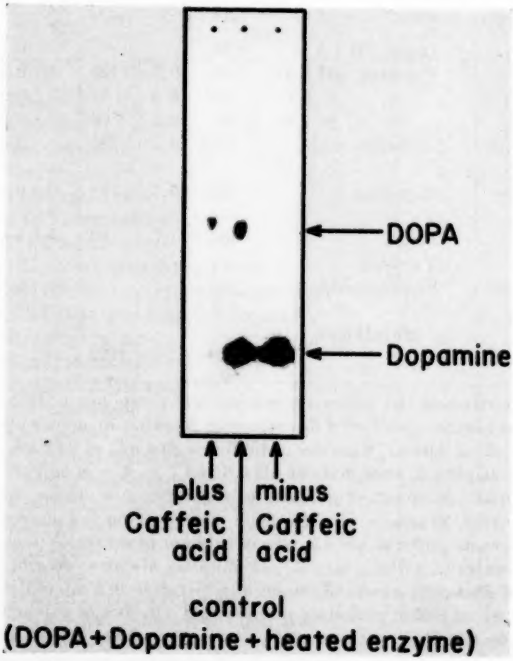


FIG. 2. Paper chromatogram showing the inhibition of the decarboxylation of dopa by caffeic acid; see "Methods" for procedure.

of this compound (Nos. 7, 8, Table VI) does not improve the activity. Some of the halogenated hydroxybenzoic acids (Table VIII, Compounds 14, 15, 16) and 3,5-dinitrosalicylic acid (Table VIII, Compound 12) are moderately active.

The method of Lineweaver and Burk (11) applied to 3,5-diiodosalicylic acid (Table VIII, Compound 16) indicated (Fig. 1) competitive inhibition at low concentrations of substrate, changing to non-competitive inhibition at higher concentrations (called "pseudoirreversibility" by Ackermann and Potter (1)). This compound does not inhibit tyrosinase or glutamic acid

decarboxylase, but inhibits 60 per cent of the activity of succinic acid dehydrogenase at 0.2 RM.

Glutathione, cysteine, and ascorbic acid, themselves without effect, were added to the reaction mixtures to prevent possible oxidation of catechol and hydroquinone derivatives to the corresponding *o*- and *p*-quinones.

Because many of the compounds were not sufficiently water-soluble for testing, propylene glycol was often used as a solvent since it had little effect on the result. Quercetin-6′-sulfonic acid (Table V, Compound 3) produces 55 per cent inhibition in propylene glycol and 67 per cent inhibition in H_2O at 0.05 RM, and the chalcone 5-(3-hydroxycinnamoyl) salicylic acid (Table IV, Compound 7) produces 66 per cent inhibition in propylene glycol and 85 per cent inhibition in H_2O at 0.01 RM.

The compounds 3-hydroxycinnamic acid and caffeic acid are fairly specific inhibitors of dopa decarboxylase (Table X). The decarboxylation of dopa by tyrosine decarboxylase is not affected, but decarboxylation of tyrosine is partially inhibited by caffeic acid at the relatively high concentration of 0.5 RM. Glutamic acid decarboxylase and histidine decarboxylase are unaffected. The oxidation of dopa by tyrosinase is slightly inhibited by caffeic acid and 3-hydroxycinnamic acid at 1.0 RM, but the oxidation of tyrosine is more strongly inhibited by caffeic acid at 1.0 RM. Succinic acid dehydrogenase is unaffected by caffeic acid. The endogenous respiration of surviving rat liver slices was not depressed by the two compounds.

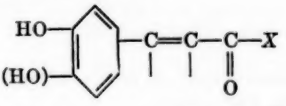
The chromatographic study (Fig. 2) shows that the dopa is completely decarboxylated to dopamine in the absence of inhibitor, but is only partly converted in the presence of caffeic acid.

SUMMARY

1. Several previously undescribed properties of mammalian dopa decarboxylase have been reported.

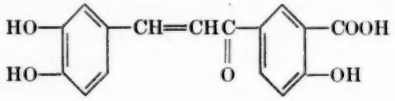
2. Three new substrates of dopa decarboxylase, *erythro*-3-hydroxyphenylserine, 2-methyl-3,4-dihydroxyphenylalanine, and 6-methyl-3,4-dihydroxyphenylalanine, have been discovered. Forty-six other phenylalanine analogues were tested and found not to be substrates.

3. Some 200 substances were tested as inhibitors of dopa decarboxylase. The best inhibitors have the structure



where *X* is OH, O-alkyl, alkyl, or aryl, the activity increasing in that

order. The most powerful inhibitor is 5-(3,4-dihydroxycinnamoyl)salicylic acid



which produces 87 per cent inhibition of the enzyme activity when its concentration is 1×10^{-3} that of the substrate.

4. The inhibition of dopa decarboxylase by 3-hydroxycinnamic acid, caffeic acid, and 5-(3,4-dihydroxycinnamoyl)salicylic acid has been shown to be competitive.

5. The compounds 3-hydroxycinnamic acid and caffeic acid are fairly specific inhibitors of dopa decarboxylase, since they do not appreciably affect other isolated enzyme systems.

6. It has been shown by paper chromatography that the inhibitor caffeic acid decreases the amount of dopamine formed by decarboxylation of dopa.

Addendum—After this manuscript was submitted, Werle and Sell (25) reported that *erythro*-3-hydroxyphenylserine and *erythro*- and *threo*-3,4-dihydroxyphenylserines are decarboxylated by mammalian tissues (*cf.* also Hartman, Pogrund, Drell, and Clark (26)). It can be seen from Table I and from the above references that these compounds are much less reactive than dopa and that *threo*-3,4-dihydroxyphenylserine (considered a non-substrate in this paper) is less than half as reactive as the erythro form.

SOURCES

1. Ivan Sorvall, Inc., Norwalk, Connecticut.
2. Precision Scientific Company, Chicago, Illinois.
3. Dr. Vera Sutter, Wadsworth General Hospital, Los Angeles, California.
4. Dr. A. B. Lerner, University of Oregon Medical School, Portland.
5. American Type Culture Collection, Washington, D. C.
6. Obtained from commercial sources.
7. Isolated by the method of Sealock, R. R., *Biochem. Preparations*, **1**, 25 (1949).
8. Dr. G. R. Bartlett, Scripps Metabolic Clinic, La Jolla, California.
9. Dr. T. A. Geissman, University of California, Los Angeles.
10. Dr. W. E. Drake, Hynson, Westcott and Dunning, Inc., Baltimore, Maryland.
11. Dr. J. Harley-Mason, University of Cambridge, England.
12. Merck Institute for Therapeutic Research, Rahway, New Jersey.
13. Dr. A. Neuberger, National Institute for Medical Research, London, England.
14. Synthesized by the method of Dickinson, W. P., and Marshall, P. G., *J. Chem. Soc.*, 1495 (1929).
15. Synthesized by the method of Sealock, R. R., Speeter, M. E., and Schweet, R. S., *J. Am. Chem. Soc.*, **73**, 5386 (1951).
16. Dr. J. M. Sprague, Sharp and Dohme, Division of Merck and Company, Inc., West Point, Pennsylvania.

17. Dr. J. H. Burckhalter, School of Pharmacy, University of Kansas, Lawrence.
18. Dr. G. J. Martin, The National Drug Company, Philadelphia, Pennsylvania.
19. Dr. C. G. Niemann, California Institute of Technology, Pasadena.
20. Dr. K. Kraft, Knoll A.-G., Ludwigshafen-am-Rhein, Germany.
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REVERSIBILITY OF ENZYMATIC TRANSGLUCOSYLATION REACTIONS*

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(Received for publication, January 17, 1955)

A number of investigators have observed the formation of new oligosaccharides during the action of transglycosidase enzymes on disaccharides. The compounds which have been shown to undergo the transglycosidation reactions include maltose (1, 2), sucrose (3, 4), lactose (5, 6), cellobiose (7), and isomaltose (2, 8). Preliminary results (8) have indicated that the conversion of isomaltose into glucose and dextrotriose by the transferring enzyme of *Aspergillus oryzae* is a reversible process. The reversibility of this conversion has now been established by the results of isotope studies. The mechanism of the reaction has been shown to involve a transfer of the glucosyl moiety of the substrates to appropriate cosubstrates and is, therefore, a transglucosylation. Details of our studies are presented in this paper.

Materials

Enzyme—The enzyme preparation of *A. oryzae* has been described previously (1). In the experiments reported here, a 1 per cent water solution of the enzyme preparation was employed.

Isomaltose (Dextrobiose)—Isomaltose, a disaccharide of 2 glucose units joined by an α-1,6-glucosidic bond, was isolated from a partial acid hydrolysate of dextran. 2 gm. of dextran were dissolved in 10 ml. of 0.1 N hydrochloric acid, and the solution was refluxed for 6 hours. Solid sodium carbonate was used to neutralize the resulting hydrolysate. Qualitative paper chromatograms showed that a series of α-1,6-oligosaccharides had been produced by this treatment. The first three members were separated and isolated by paper chromatography as described previously (9). The yield of isomaltose was 0.25 gm. The specific rotation of the isomaltose was +116° (*c*, 2 per cent in water); literature value, +120° (10, 11). On paper chromatography the preparation gave one distinct spot with an R_F value identical to that for pure isomaltose.[1] Glucose was the only hydro-

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[1] Pure isomaltose for chromatographic comparisons was kindly supplied by Dr.

lytic product detectable on partial and complete acid hydrolysis of the compound.

100 mg. of the isomaltose were dissolved in 5 ml. of water. 30 mg. of *o*-phenylenediamine, 180 mg. of phenylhydrazine hydrochloride, and 0.1 ml. of glacial acetic acid were added. The mixture was heated at 100° for 6 hours. On cooling the reaction mixture, a yellow precipitate was obtained. The precipitate was collected on a filter and air-dried. It was crystallized from 5 ml. of hot *n*-propyl alcohol. X-ray diffraction patterns[2] of the crystalline compound and of the flavazole (1) from pure isomaltose were identical.

Dextrotriose—The dextran trisaccharide, dextrotriose, consists of 3 glucose residues united by two α-1,6 bonds. It was isolated in pure form from a partial acid hydrolysate of dextran by the chromatographic procedure. 0.20 gm. of dextrotriose was obtained from the dextran hydrolysate described above. On paper chromatography the preparation gave one distinct spot which corresponded in position to the third member of the dextran series. Partial acid hydrolysis converted the compound to glucose and isomaltose, while prolonged hydrolysis converted it completely to glucose. The specific rotation of the compound is $+140°$ (*c*, 0.8 per cent in water) and is in agreement with a literature value of $+145°$ (11).

Other Materials—"Panose" (4-α-isomaltosyl-D-glucose) (12, 13) was kindly provided by Dr. S. C. Pan, E. R. Squibb and Sons, New Brunswick, New Jersey. C^{14}-Glucose uniformly labeled was purchased from the Nuclear Instrument and Chemical Corporation, Chicago, Illinois. Samples of dextran were provided by Dr. A. Jeanes and Dr. R. J. Dimler, Northern Utilization Research Branch, United States Department of Agriculture, Peoria, Illinois.

Methods and Results

Digest of Isomaltose—4 mg. of pure isomaltose were dissolved in 0.05 ml. of water and mixed with 0.05 ml. of transglucosylase solution of *A. oryzae*. An aliquot of the digest was placed on a paper strip as soon after addition of the enzyme as possible and heated immediately at 100° for 5 minutes in order to arrest enzyme action. Subsequent samples were obtained from the digest after reaction periods of 12, 24, and 48 hours. The reducing sugars in these aliquots were separated and identified by paper chromatography. A photograph of the chromatogram of the isomaltose digests is reproduced in Fig. 1. It is readily seen that the isomaltose undergoes a

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[2] We are grateful to the University Research Council for use of the x-ray equipment and to Mr. W. C. Robison, Electrical Engineering Department, University of Nebraska, for operation of the equipment.

disproportionation reaction, yielding glucose and a higher molecular weight oligosaccharide. Chromatographic evidence indicates that the new

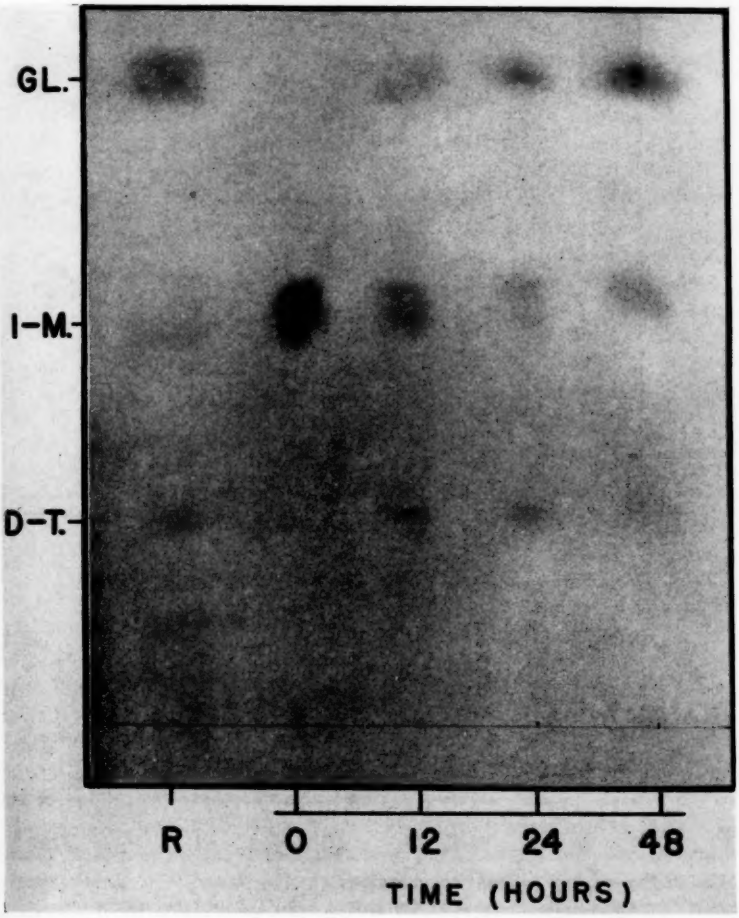

FIG. 1. A multiple ascent paper chromatogram of isomaltose digest at several stages of enzymolysis. *GL.*, glucose; *I-M.*, isomaltose; *D-T.*, dextrotriose; and *R*, reference dextran oligosaccharides.

oligosaccharide is dextrotriose. The concentration of the glucose and dextrotriose increased with reaction time, while the concentration of isomaltose decreased.

Digest of C^{14}-Glucose and Dextrotriose—3 mg. of C^{14}-glucose and 6 mg. of

dextrotriose were dissolved in 0.05 ml. of water and mixed with 0.05 ml. of enzyme solution. Samples of the digest were placed on two separate paper

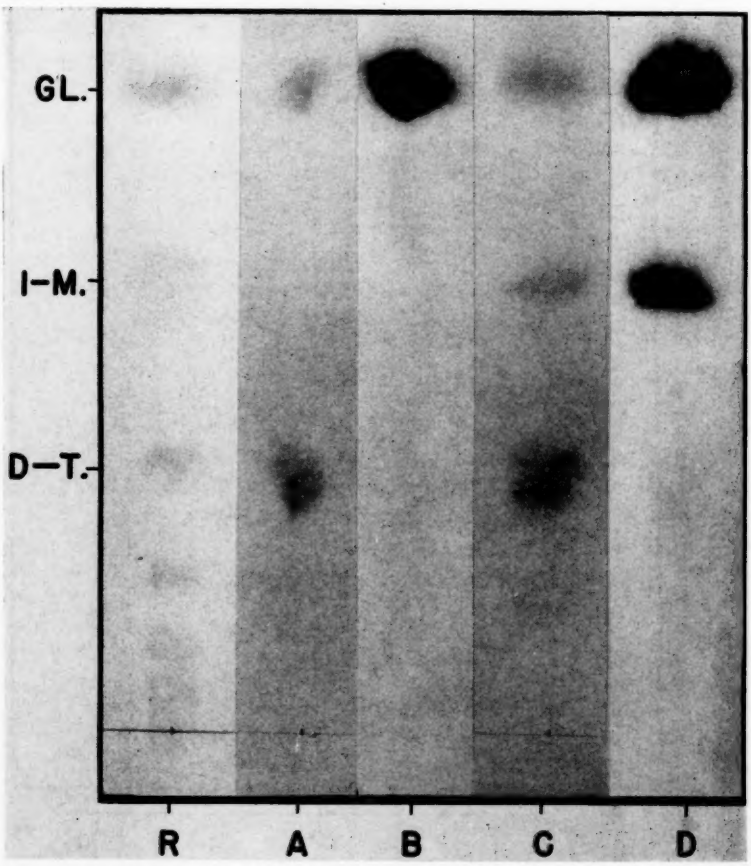

FIG. 2. Paper strips (*A* and *C*) and radioautographs (*B* and *D*) of the products in enzymolysate of C^{14}-glucose and dextrotriose at 0 and 24 hour reaction periods. *GL.*, glucose; *I-M.*, isomaltose; *D-T.*, dextrotriose; and *R*, reference dextran oligosaccharides.

strips for 0, 12, 24, and 48 hours. The paper strips were developed in *n*-butyl alcohol-pyridine-water (6:4:3 by volume) solvent. The finished chromatograms were placed in contact with Kodak "no screen" x-ray film for 6 days. The radioactive components in the digest showed up as dark

spots on the developed film. One of the paper chromatograms was sprayed with copper sulfate reagent (14) to locate the position of the reducing sugars. Photographs of strips from the chromatogram for the 0 and 24 hour periods are reproduced in Fig. 2. The radioautographs of these strips are also shown. Inspection of Fig. 2 reveals that glucose and dextrotriose were the reducing compounds in the digest at 0 hour and that at 24 hours a new reducing compound (isomaltose) was produced in the digest. The radioautograph shows that the isomaltose was radioactive.

Digest of C^{14}-Glucose and Isomaltose—2 mg. of C^{14}-glucose and 6 mg. of isomaltose were dissolved in 0.05 ml. of water. 0.05 ml. of the enzyme solution was added. Samples of 0.01 ml. of the digest were placed on three paper strips after reaction times of 0, 12, and 24 hours and treated as in the previous experiment. One of the developed chromatograms was sprayed with copper sulfate reagent, while the other two were placed in contact with

TABLE I

Radioactivities of Compounds in Enzymolysates of C^{14}-Glucose and Isomaltose

Compound	Time		
	0 hr.	12 hrs.	24 hrs.
	c.p.m.	*c.p.m.*	*c.p.m.*
Glucose	2302	1990	1415
Isomaltose	5	256	630
Dextrotriose	2	15	205

x-ray film. The areas at which the reducing compounds appeared on the sprayed chromatogram were cut from the paper and counted with a thin walled mica tube and Tracerlab scaler. The radioactivities of the compounds in the digest are recorded in Table I.

Digest of C^{14}-Glucose and Panose (4-α-Isomaltosyl-D-glucose)—4 mg. of C^{14}-glucose and 20 mg. of panose were dissolved in 0.25 ml. of water, then mixed with 0.25 ml. of enzyme solution. Samples of 0.1 ml. were taken at 0, 6, 24, and 48 hours, heated for 5 minutes at 100°, and examined for reaction products by the methods of the previous experiment. Radioactivities of the products were measured directly on the paper chromatogram. The compounds in the digest have been tentatively identified by their R_F values. These and the radioactivities of the compounds are presented in Table II. The C^{14}-glucose included in the digest was incorporated predominantly into isomaltose and perhaps to a slight extent into maltose and dextrotriose. Visual comparisons of the intensities of the carbohydrate spots on the chromatogram showed that the concentration of the maltose passed through a maximum at 6 to 12 hours and that the concentrations of the isomaltose

and higher oligosaccharides increased, while the concentration of panose decreased with time.

Isolation and Degradation of C^{14}-Isomaltose—The radioautographs of the digests of C^{14}-glucose-isomaltose and C^{14}-glucose-dextrotriose were used for locating the areas of the chromatogram at which the C^{14}-isomaltose was present. The C^{14}-isomaltose was extracted from these areas with water into separate test-tubes. 3 mg. of non-active isomaltose were added to each tube. The resulting solutions were taken to dryness under vacuum. The residues were redissolved in 0.05 ml. of 0.1 N potassium hydroxide. Aliquots of the solutions were placed on two different paper strips. A crystal of iodine was added to the reaction mixtures, and oxidation of the isomaltose was allowed to proceed at room temperature for 24 hours.

TABLE II

Radioactivities of Compounds in Enzymolysates of C^{14}-Glucose and Panose

Compound	R_F value	Time			
		0 hr.	6 hrs.	24 hrs.	48 hrs.
		c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
Glucose	0.81	3840	3522	3360	3125
Maltose	0.57	12	44	52	36
Isomaltose	0.43	35	292	390	468
Panose	0.27	18	15	18	21
Dextrotriose	0.16	6	18	34	47
Tetrasaccharide	0.08	22	32	27	30

Samples of the oxidized mixtures were placed on the chromatogram alongside the initial spots. Next, 0.05 ml. of 0.5 N hydrochloric acid was added to the oxidation mixture, and the solutions were heated in a sealed tube for 3 hours at 100°. Aliquots of the hydrolysates were also placed on the paper strips. The paper strips were developed in *n*-butyl alcohol-pyridine-water system (6:4:3 by volume) and sprayed with copper sulfate solution (14). The areas at which reducing sugars were present were counted, while the remainder of the chromatogram was scanned for radioactivity. The following results were obtained for the degradation of the C^{14}-isomaltose from the digest of C^{14}-glucose and isomaltose: The R_F value of the isomaltose in the initial sample was 0.36, and its activity was 490 c.p.m. The isomaltobionic acid produced on oxidation of the C^{14}-isomaltose was located at R_F 0.05 and had an activity of 412 c.p.m. Hydrolysis of the isomaltobionic acid yielded non-active glucose (R_F value 0.78) and radioactive gluconic acid (R_F value 0.09 and activity 175 c.p.m). Similar values were obtained for the degradation of the C^{14}-isomaltose from the digest of C^{14}-glucose and dextrotriose.

DISCUSSION

The results in Figs. 1 and 2 show that the transferring enzyme of *A. oryzae* catalyzes glucosyl exchange reactions among glucose, isomaltose, and dextrotriose. C^{14}-Glucose was a valuable cosubstrate for following the course of the reactions and, when used with the substrates, was incorporated into isomaltose. Degradation of the C^{14}-isomaltose showed that it was labeled in the glucosido unit. In order to produce C^{14}-isomaltose labeled in this manner from these compounds, a transfer of the glucosyl unit of the substrate to the 6 position of free C^{14}-glucose must have occurred. The name transglucosylation has been suggested for reactions of this type (15).

If a glucosyl-enzyme complex (G·E) is formulated as an intermediate in the reaction, the mechanism of the transglucosylation of isomaltose may be visualized as a two-step process (Equations 1 and 2).

$$G'G'' + E \rightleftarrows G'\cdot E + G \quad (1)$$

$$G'\cdot E + G'G'' \rightleftarrows G'G'G'' + E \quad (2)$$

G, G′, and G″ represent glucose, the glucosyl unit, and the glucosido unit substituted at the 6 position, and E represents an enzyme molecule. The production of C^{14}-isomaltose from C^{14}-glucose and dextrotriose (Fig. 2) establishes the reversibility of this process. Additional evidence for reversibility was forthcoming from the experiment with C^{14}-glucose and isomaltose. From these substrates the enzyme synthesized C^{14}-isomaltose, most probably by a reversal of Equation 1 of the mechanism.

Since panose (4-α-isomaltosyl-D-glucose) possesses a terminally linked α-1,6-glucose unit and is acted upon by the transferring enzyme of *Aspergillus niger* (2), it was tested as a possible substrate for the *A. oryzae* enzyme. The reducing sugars produced during the action of the enzyme on panose and C^{14}-glucose are listed in Table II, and radioactivities of the products are also recorded there.

A reaction sequence by which these products may arise in the mixture is shown in the accompanying equations.

$$G'G'G''' + E \rightleftarrows G'\cdot E + G'G''' \quad (3)$$

$$G'\cdot E + G \rightleftarrows G'G'' + E \quad (4)$$

$$G'\cdot E + G'G'' \rightleftarrows G'G'G'' + E \quad (5)$$

$$G'\cdot E + G'G'G''' \rightleftarrows G'G'G'G''' + E \quad (6)$$

The G‴ represents a glucosido unit substituted in the 4 position, and the other notations are the same as in Equations 1 and 2. The isotope experiment showed that C^{14}-glucose was incorporated in isomaltose (Equation 4) which in turn functioned as a cosubstrate in Equation 5 to yield radioactive dextrotriose. The concentrations of dextrotriose and of the tetrasaccharide

(G′ G′ G′ G‴) were always low in the reaction mixture. Since the enzyme converts α-1,4 bonds to α-1,6 bonds (1), the maltose produced in Equation 3 is converted to α-1,6-glucosyl oligosaccharides and disappears from the mixture. Whereas the transferring enzyme of *A. niger* has been found by Pan *et al.* (2) to effect a reversible synthesis of maltose from glucose and panose, little, if any, maltose was synthesized from these substrates by the transferring enzyme of *A. oryzae*.

SUMMARY

The transferring enzyme of *Aspergillus oryzae* has been shown to effect a transglucosylation of α-1,6-glucosyl oligosaccharides. Radioactive isotopes have been used to demonstrate that the transglucosylation reactions are reversible.

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THE METABOLISM OF ASCORBIC ACID-1-C^{14} AND OXALIC ACID-C^{14} IN THE RAT*

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(Received for publication, January 20, 1955)

It is known that oxalic acid is a product of ascorbic acid metabolism in the guinea pig (1) and rat (2), and is formed in man from unidentified precursors (3). Oxalates are also of interest because of their occasional toxicity and unexplained deposition. Ascorbic, dehydroascorbic, and 2,3-diketogulonic acids are known to give rise to oxalic and threonic acids in approximately quantitative yields by chemical oxidation (4, 5).

Although diketogulonic acid has no appreciable antiscorbutic value, it has been reported in the tissues and urine of the guinea pig (6, 7) and rat (8). Damron, Monier, Roe, and Weiss (7, 8) observed that under normal conditions dehydroascorbic and diketogulonic acids were formed only to a small extent from ascorbic acid *in vivo*, except when the animals were exposed to low temperatures.

Studies of ascorbic acid-1-C^{14}, dehydroascorbic acid-1-C^{14}, 2,3-diketogulonic acid-1-C^{14}, and oxalic acid-C^{14} metabolism under approximately normal conditions in the rat are reported in the present paper. The results show that the 6-carbon acids give rise to appreciable quantities of urinary oxalate. Oxalic acid is not oxidized appreciably to carbon dioxide *in vivo*, however, nor is there evidence of its entering metabolic pathways other than excretion. The data indicate a half life for ascorbic and oxalic acids, respectively, as 3.6 and 2.5 days in the rat.

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† The data presented in this manuscript constitute a portion of the thesis submitted by Constance O'H. Curtin in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Faculty of Pure Science, Columbia University. Present address, Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Illinois.

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EXPERIMENTAL

Preparation of Labeled Compounds—Ascorbic acid-1-C^{14} was prepared[1] by the methods reported by Burns and King (9) and Salomon, Burns, and King (10).

Dehydroascorbic acid-1-C^{14} solution was prepared immediately before use by oxidation of ascorbic acid-1-C^{14} with an equivalent quantity of quinone (11). Less than 2 per cent of the injected solution of dehydroascorbic acid-1-C^{14} was in the form of ascorbic acid-1-C^{14}, as shown by spectrophotometric analysis and paper chromatography (12).

Calcium 2,3-diketogulonate-1-C^{14} was prepared from ascorbic acid-1-C^{14} (100 mg.) by the addition of an equivalent amount of potassium iodate in solution, followed by opening of the lactone ring in base according to the method of Penney and Zilva (5) with the following modifications. The dropwise addition of N sodium hydroxide was continued only until the pH of the dehydroascorbic acid solution reached 7.5. Hydriodic acid (1 N) was added immediately until the pH returned to 3. Ethanol was added to a final concentration of 80 per cent by volume before calcium iodide was added (13), and the pH was again adjusted to 3. After 70 per cent of the equivalent amount of calcium iodide was added, ethanol was again added to a final concentration of 80 per cent by volume. After 30 minutes at room temperature, 10 to 15 ml. of peroxide-free ether were added. The temperature of the solution was brought to 7–8° and another small portion of ice-cold ether was added. The precipitate was filtered at 7° on a sintered glass funnel and washed with cold ethanol, followed by cold ether. After air drying for 10 minutes, the salt was kept at −10°. Yield, 66 per cent. Calculated for $C_{12}H_{14}O_{14}Ca \cdot 8H_2O$, C 25.44, H 5.34; found, C 25.81, H 5.44.

The specific optical rotation found for calcium diketogulonate·$8H_2O$ was $[\alpha]_D^{20}$ −8.6°, in agreement with the value reported by Penney and Zilva. The calculated value for diketogulonic acid therefore was $[\alpha]_D^{20}$ −12.6°.

The specific activity of the calcium diketogulonate-1-C^{14}, 6.05×10^7 c.p.m. per mmole, agreed within the limits of error with that of the original ascorbic acid-1-C^{14} of known radioactive purity, 5.34×10^7 c.p.m. per mmole. Also a single diketogulonic acid-1-C^{14} spot with a differing R_F from ascorbic acid, dehydroascorbic acid, and oxalic acid was found on paper chromatograms. The solvent systems used were butanol-acetic acid-water, isobutyric acid-water, and butanol-ethanol-water (12).

Ascorbic acid 2,4-dinitrophenylosazone was prepared from non-radioactive calcium diketogulonate·$8H_2O$ (14) without preliminary use of the exchange resin or oxidation; m.p. 291–294°, with decomposition; m.p.

[1] The ascorbic acid-1-C^{14} used was prepared by Dr. John J. Burns and Dr. Lothar L. Salomon.

291–294°, with decomposition,[2] of ascorbic acid 2,4-dinitrophenylosazone from synthetic ascorbic acid.

The calcium salts of ascorbic acid or dehydroascorbic acid were not precipitated under the conditions used for calcium diketogulonate-1-C^{14} preparation.

Oxalic acid-C^{14} was obtained from the Isotopes Division of the Atomic Energy Commission at Oak Ridge. It had been prepared by fusion of potassium carbonate and potassium in the presence of sand, and shown to have no radioactive impurities by radioautographs of paper chromatograms in the Radiation Laboratory of the University of California, Berkeley, California.

Injection Experiments—Adult male rats of the Wistar strain, ranging in weight from 270 to 355 gm., were given a diet of evaporated milk and water for 2 weeks prior to each experiment and during the experiments.

Amounts of the labeled compounds given by intraperitoneal injection in 0.6 ml. of solution ranged from 1.5 to 5.9 mg. and exhibited from 2.02×10^5 c.p.m. to 2.04×10^7 c.p.m., except in one oxalic acid-C^{14} experiment in which 0.08 mg. with a total radioactivity of 9.14×10^5 c.p.m. was injected.

Immediately after injection the rats were placed in a metabolism chamber (15, 16) through which carbon dioxide-free air was drawn. Respiratory carbon dioxide was collected at intervals during 24 hours in 2.5 N sodium hydroxide of known carbonate content. The 24 hour urine samples were collected in 5 ml. of 3 per cent oxalic acid (15) since this acid is most satisfactory as a stabilizing agent. In the oxalate-C^{14} injection experiments, carrier oxalate was added at the end of 24 hours because of the high specific activity. To the collection bottles were added 5 ml. of 4 N hydrochloric acid in addition to carrier oxalate. At the end of 24 hours the animals were decapitated. When samples were collected every 24 hours for 7 to 10 days after injection, the animals were sacrificed with ether, since tissue analyses were not performed. The feces were dried for several days over Drierite in a vacuum desiccator and powdered.

Preparation of Samples for Radioactivity Determinations—Oxalic acid was isolated from the urine as calcium oxalate monohydrate (1) and from the liver, kidneys, and adrenals by the same method after a trichloroacetic acid protein precipitation. The specific activity of the calcium oxalate monohydrate remained constant after reprecipitation. Urinary ascorbic acid was isolated as ascorbic acid 2,4-dinitrophenylosazone (14).

For an estimation of the C^{14} present in urea, dixanthydryl urea was isolated by the method of Fosse, Robyn, and Francois as modified by Allen

[2] All melting points were taken on a micro melting point apparatus, Arthur H. Thomas Company, Philadelphia, unless marked as corrected.

and Luck (17). The melting point of the isolated dixanthydryl urea was 267–268° (corrected) with decomposition.

After removal of the tissues necessary for the particular experiment, the total carcass was ground, digested in hot alcoholic potassium hydroxide, and brought to a known volume. The tissues were homogenized in a Waring blendor or a Potter-Elvehjem homogenizer in the presence of 75 mg. of carrier oxalic acid. Aliquots of the injection solutions and all tissue and urine samples were dried in a vacuum desiccator over phosphorus pentoxide and converted to barium carbonate by wet combustion (18, 19). The $C^{14}O_2$ was obtained from calcium oxalate, dixanthydryl urea, and feces by wet combustion also. The respiratory carbon dioxide was precipitated as barium carbonate (16) and samples were collected on filter paper disks (16, 20).

Radioactivity Measurement—C^{14} measurements were made on the barium carbonate disks in a Q gas counter.[3] The observed count was corrected for background and self-absorption (21). The standard error (22) for the final respiratory carbon dioxide values was not greater than ±13 per cent after ascorbic acid-1-C^{14} injections. For the respiratory carbon dioxide hourly values, after injections of dehydroascorbic acid-1-C^{14} and diketogulonic acid-1-C^{14}, the over-all standard error was less than ±17 per cent through the 9th and 5th hours, respectively.

RESULTS AND DISCUSSION

End-Products of Metabolism—The peak of respiratory $C^{14}O_2$ yield after injection of dehydroascorbic acid-1-C^{14} and diketogulonic acid-1-C^{14} occurred during the 1st hour (Fig. 1). In contrast, the maximal (lower) $C^{14}O_2$ yield after ascorbic acid-1-C^{14} injection occurred between the 4th and 6th hours. The recovery of injected radioactivity in the respiratory carbon dioxide at the end of 24 hours (Table I) was 19 per cent (average) from the rats injected with ascorbic acid-1-C^{14}. The average value (17 per cent) from the diketogulonic acid-1-C^{14} tests at pH 7 was similar, and demonstrated the capacity of the rat to metabolize the substance readily. When dehydroascorbic acid-1-C^{14} was given, the average value was 29 per cent, and when diketogulonic acid-1-C^{14} was injected at a pH of 3, the average value was 46.7 per cent. This difference in utilization from solutions of different pH values may have been induced by partial lactonization.

The finding that a higher proportion of the dose appeared in the respiratory carbon dioxide after dehydroascorbic acid-1-C^{14} injection than after ascorbic acid-1-C^{14} injection does not establish that dehydroascorbic acid is on a pathway of ascorbic acid oxidation to carbon dioxide. It is likely,

[3] Scaling unit model No. 163, Nuclear Instrument and Chemical Corporation, Chicago, Illinois.

however, that some of the ascorbic acid is converted to dehydroascorbic acid as a normal intermediate product (8, 23, 24).

The percentage of the injected quantity excreted as oxalate during the first 24 hours after dehydroascorbic acid-1-C^{14} and diketogulonic acid-1-C^{14} injections was approximately 5 times the corresponding value found in the ascorbic acid-1-C^{14} tests (Table I). The marked difference in oxalate excretion after injecting diketogulonic acid-1-C^{14} at pH 3 and pH 7 (Table I) was not expected. A solution of diketogulonic acid, brought to pH 7 and

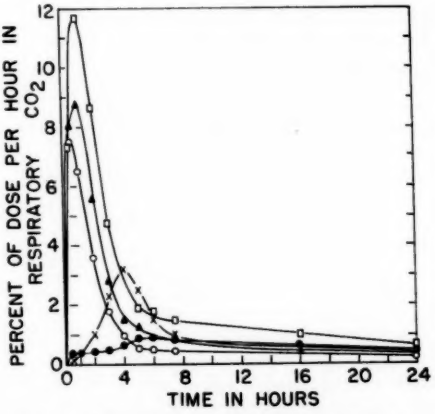

FIG. 1. Hourly rate of C^{14} excretion in respiratory CO_2 after intraperitoneal injection of (●) ascorbic acid-1-C^{14} at pH 3; (×) ascorbic acid-1-C^{14} at pH 7; (▲) dehydroascorbic acid-1-C^{14} at pH 3; (○) 2,3-diketogulonic acid-1-C^{14} at pH 7; and (□) 2,3-diketogulonic acid-1-C^{14} at pH 3.

returned to pH 3 after several minutes, still permitted precipitation of calcium diketogulonate in good yield.

Diketogulonic acid-1-C^{14} gave rise to both $C^{14}O_2$ and urinary oxalate after injection into the rat, without appreciable formation of ascorbic or dehydroascorbic acid, but the data do not eliminate 2,3-diketogulonic acid-1-C^{14} from consideration as a possible metabolic product of ascorbic acid. The 2,4-dinitrophenylosazone of ascorbic acid isolated from the 24 hour urine sample of a rat after injection with diketogulonic acid-1-C^{14} did not contain a significant yield of C^{14}.

Dixanthydryl urea was isolated from the first 24 hour urine of a rat after injection with diketogulonic acid-1-C^{14}. Not more than 1.64 per cent of the dose could be accounted for in 79 mg. of urea. This yield is regarded as insignificant because of the probable recycling of some C^{14} from carbon dioxide.

Approximately 42 per cent of the total count found in the first 24 hour urine samples after injection of ascorbic acid-1-C^{14} was accounted for as unchanged ascorbic acid plus oxalic acid (Table I). The ascorbic acid alone contained 12 per cent of the initial C^{14}. When ascorbic acid-1-C^{14} was added to non-radioactive urine to test for both recovery and conversion to other products, 95 to 100 per cent of the added radioactivity was recovered as the 2,4-dinitrophenylosazone. The total radioactivity found in

TABLE I

Distribution of Radioactivity 24 Hours after Injection of Rats with Ascorbic Acid-1-C^{14}, Dehydroascorbic Acid-1-C^{14}, and 2,3-Diketogulonic Acid-1-C^{14}

The values are given in per cent of dose.

Compound injected (1-C^{14})	Urinary oxalate	Respiratory CO_2	Total urine	Urinary ascorbic acid	Liver	Feces	Remaining in carcass	Total accounted for
Ascorbic acid*	2.2†	16.2	30.3		7.9	0.8	51.4	107
" "	2.2	21.3	35.0	12.5	4.8	0.1	42.8	104
Dehydroascorbic acid*	7.5‡	28.2						
" " *	9.7‡	29.8						
2,3-Diketogulonic acid	8.1	19.4	70.4		—§	—	—	89.8
" "	13.3	15.2	83.3		—	0.2	—	98.7
" " *	14.9	46.7	43.6	<0.48	—	0.5	—	90.8

* These compounds were injected at pH 3. All others were injected at pH 7.

† Two independent injection experiments with ascorbic acid-1-C^{14} at pH 3 yielded values of 2.3 and 2.1 per cent of the C^{14} dose in urinary oxalate in 24 hours.

‡ These samples were counted directly as calcium oxalate monohydrate and the count as barium carbonate was calculated by using an experimentally determined factor, 0.77 ± 0.08, which was the ratio of the counts per minute as barium carbonate over the counts per minute of the same sample as calcium oxalate monohydrate.

§ Spaces filled in by a dash indicate that the aliquot radioactivity was between background and 4 c.p.m. above background.

the animal and in metabolic products[4] agreed fairly closely with the quantity of C^{14} injected (Table I).

Oxalic Acid—When oxalic acid-C^{14} was injected intraperitoneally into the rat, less than 0.4 per cent of the dose was detected in the respiratory carbon dioxide within 24 hours, which is in general agreement with the findings of Weinhouse and Friedmann of approximately 1 per cent in each of two rats (25). Therefore the occurrence of C^{14} in the CO_2 after injections of ascorbic acid-1-C^{14}, dehydroascorbic acid-1-C^{14}, and diketogulonic

[4] The radioactivity in the muscle sample measured was between background and 4 c.p.m. above background. If the total radioactivity in body muscle is calculated on the basis of the observed sample count, the muscle contained more total radioactivity than any other tissue.

acid-1-C^{14} probably did not involve an intermediate conversion of these compounds to oxalate-C^{14}. All radioactivity excreted in the urine and feces within the range of measurement was present as unchanged oxalic acid (Table II). Hence it is probable that the radioactivity found as oxalate after injection of the labeled compounds accounted for the approximate total conversion of these compounds to oxalate. In a control experiment in which oxalic acid-C^{14} was added to non-radioactive urine, 97.2 per cent of the added count was recovered.

The failure of animal tissues to utilize oxalate is of interest in relation to analogous observations in the tobacco plant (26). Oxalate thus appears to

TABLE II

Distribution of Radioactivity after Intraperitoneal Injection of Oxalic Acid-C^{14} into Rat

The values are given in per cent of dose.

Sample No.	Urine		Urinary oxalate		Feces		Feces as oxalate	
	Rat R-17*	Rat R-31†	Rat R-17	Rat R-31	Rat R-17	Rat R-31	Rat R-17	Rat R-31
1	64.4	58.0	66.3	57.5	14.2	3.19	14.0	3.18
2				5.79		2.91		3.04
3		2.67				1.76		
4		1.58				1.01		
10		0.27	0.29					

* Rat R-17 was injected intraperitoneally with sodium oxalate-C^{14} at pH 7. The dose was 9.14×10^5 c.p.m. in 0.10 mg. of sodium oxalate-C^{14}.

† Rat R-31 was injected intraperitoneally with oxalic acid-C^{14} at pH 2. The dose was 2.04×10^6 c.p.m. in 2.40 mg. of oxalic acid-C^{14}. The urinary oxalate values for the 5 to 9 day samples as a basis for half life calculations were, respectively, 1.02, 0.84, 0.58, 0.53, and 0.35.

be remarkably inactive as a product of metabolism. A question whether the injected oxalate-C^{14} penetrated the cells sufficiently to be fully mixed with endogenous oxalate appears to be partially answered by the distribution of radioactivity in the tissues of the rat 10 days after oxalic acid-C^{14} injection. The bone chips, for example, even after vigorous treatment with potassium hydroxide and washing, retained a substantial amount of radioactivity. The counts per minute per gm. of washed tissue 10 days after injection were as follows: scraped tibia and fibula 11,400, bone chips 4500, spleen 2200, adrenals 1900, kidney 860, liver 800, remainder of carcass 1800.

The excretion of labeled oxalic acid after injection of ascorbic acid-1-C^{14} was followed in two experiments for 7 and 10 days. The total urinary oxalate-C^{14} within 10 days represented 4.9 per cent of the initial quantity.

The counts in the excreted oxalate each day were plotted against time on semilogarithm paper (Fig. 2). From the straight line obtained, the half life of ascorbic acid-1-C^{14} was found to be 3.6 days. The error of each measurement was less than ± 10 per cent. The error of the slope of the line was ± 4.1 per cent. The value obtained (3.6 days) as a measure of the turnover rate of ascorbic acid is in general agreement with the reported values, based upon direct measurement of ascorbic acid-1-C^{14} excretion (27). The body pool of ascorbic acid in the rat has been calculated (27) as 24 to 43 mg. From this and the half life reported above, 3.6 days, it was calcu-

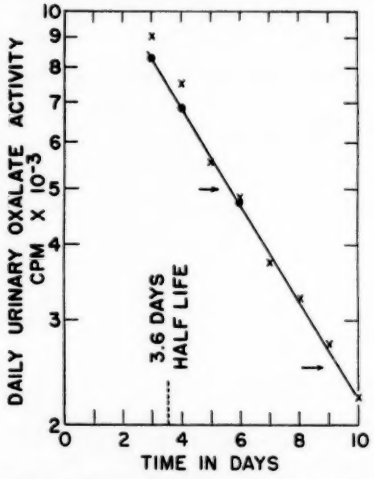

FIG. 2. Daily rate of total C^{14} excretion in urinary oxalate after intraperitoneal injection of ascorbic acid-1-C^{14} into the rat plotted on semilogarithm paper (two experiments).

lated that approximately 5 to 8 mg. of ascorbic acid were synthesized by the rat per day. Similar treatment of rat data has been reported previously (28, 29).

The excretion of labeled oxalic acid after injection of oxalic acid-C^{14} was also followed. The half life of oxalic acid was found to be 2.5 days (Table II). The error of each measurement was less than ± 10 per cent and the error of the slope of the line was ± 7.7 per cent.

Control experiments were carried on to test the possible conversion in the urine of ascorbic, dehydroascorbic, and diketogulonic acids to oxalate during the 24 hour collection period and subsequent oxalate isolation. A 24 hour sample of non-radioactive rat urine was collected under the same conditions as in the injection experiments. Only 0.4 per cent (three experi-

ments) of added ascorbic acid-1-C^{14} and 0.6 per cent (two experiments) of added dehydroascorbic acid-1-C^{14} radioactivity appeared as oxalate. These figures are small compared with the percentage found as urinary oxalate after ascorbic acid-1-C^{14} injection. When diketogulonic acid-1-C^{14} was tested as a control, 4 per cent (two experiments) of the total count added appeared as oxalate in 24 hours. Hence, if ascorbic acid gave rise to this substance as an intermediate, it might become a significant source of oxalate. Negligible amounts of radioactivity were found as oxalate in the liver 24 hours after injection of a rat with ascorbic acid-1-C^{14}, although the total C^{14} retained in the liver was 4.8 per cent of the quantity injected.

SUMMARY

1. The conversions of ascorbic acid-1-C^{14} and dehydroascorbic acid-1-C^{14} to respiratory $C^{14}O_2$ in the rat were found to be approximately 19 and 29 per cent of injected C^{14} respectively within 24 hours. Values for 2,3-diketogulonic acid-1-C^{14} conversion to $C^{14}O_2$ were 17 per cent at pH 7 and 47 per cent at pH 3. The conversions of ascorbic acid-1-C^{14}, dehydroascorbic acid-1-C^{14}, and diketogulonic acid-1-C^{14} to urinary oxalate-C^{14} were approximately 2, 9, and 12 per cent of injected radioactivity, respectively, within 24 hours.

2. 2,3-Diketogulonic acid-1-C^{14} injected into the rat was not converted to ascorbic acid-C^{14}.

3. Not more than 0.4 per cent of radioactivity injected into the rat as oxalic acid-C^{14} was found as labeled carbon dioxide within 24 hours. No evidence was found for the conversion of oxalate-C^{14} to other compounds in the rat.

4. The distribution of radioactivity in rat tissues 10 days after injection with oxalic acid-C^{14} indicated that oxalate-C^{14} was widely distributed in rat tissue, with a relatively high concentration in bone.

5. The half life of ascorbic acid, 3.6 days, was estimated from the amount of C^{14} excreted daily in urinary oxalate after ascorbic acid-1-C^{14} injection. The rate of ascorbic acid synthesis in the rat was estimated to be 5 to 8 mg. per day.

6. The half life of oxalic acid, 2.5 days, was estimated by measuring the amount of radioactivity in the urine daily after oxalic acid-C^{14} injection into the rat.

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THE BIOSYNTHESIS OF HYALURONIC ACID BY GROUP A STREPTOCOCCUS

IV. RÔLE OF GLUCOSONE AS AN INTERMEDIATE IN THE SYNTHESIS OF GLUCOSAMINE*

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That glucosamine arises from glucose without previous scission of the carbon chain has been indicated by studies of the biosynthesis of the glucosamine portion of the hyaluronic acid (HA) molecule by group A streptococcus (1, 2) and of the formation of blood glucosamine in rats (3, 4). Relatively little information is available regarding the mechanism of this reaction, although Lowther and Rogers (5), in a preliminary report of studies utilizing resting suspensions of hemolytic streptococci, found evidence that glutamine or a mixture of glutamic acid and NH_4^+ is necessary for the synthesis of HA. More recently, Leloir and Cardini (6) found evidence that glucosamine-6-phosphate is formed by the reaction of the amide group of glutamine and glucose-6-phosphate in extracts of *Neurospora crassa*.

Glucosone, because of its structural relationship to glucose, has been the subject of a variety of investigations regarding its possible relationship to the metabolism of glucose. The earlier literature regarding both biological and chemical studies of this compound has been reviewed in detail by Becker *et al.* (3, 4, 7, 8).

Utilizing glucose-1-C^{14} and glucosone-1-C^{14}, Becker and Day (3, 4) found that a greater portion of the radioactivity of the glucosone than that of glucose is converted to blood glucosamine in the rat and postulated that glucosone is an intermediate in the synthesis of glucosamine. In a study of the biosynthesis of HA by group A streptococci, Topper and Lipton (2) observed that the addition of inactive glucosone to a medium containing glucose-1-C^{14} resulted in a decrease in the labeling of the isolated hyaluronic acid and concluded that, "The present findings suggest that the dicarbonyl compound may indeed lie in the reaction sequence: glucose to glucosone to glucosamine."

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Previous studies from this laboratory have been concerned with the mechanism of biosynthesis of HA (1, 9). A study of the possible rôle of glucosone led to conclusions at variance with those discussed above. It is the purpose of the present report to present these data.

Methods

The organism, medium, and methods for isolation and degradation of HA were identical with those previously described with the exception that in the last experiment (Table II) an enzymatic hydrolysate of casein was substituted for the acid hydrolysate previously utilized. This modification has been found recently to afford more reproducible growth and better HA production.

Glucosone-1-C^{14} was prepared from glucose-1-C^{14} by treatment of phenylglucosazone with pyruvic acid by modification of the method of Becker (7, 8). The modification involved the use of 1:1 isopropyl alcohol as the solvent and refluxing the mixture for 1.5 hours. These changes permitted the more convenient preparation of larger amounts of material in a yield of 50 per cent. The great lability of glucosone makes adequate identification of this compound difficult; however, paper chromatography by the method of Flood *et al.* (10) indicated that the compound obtained migrated as a single spot. The glucose-1-C^{14} was obtained through the courtesy of Dr. H. S. Isbell, National Bureau of Standards. Uniformly labeled glucosone was prepared from uniformly labeled glucose obtained from Dr. Norbert J. Scully of the Argonne National Laboratory.

EXPERIMENTAL

The first experiments, illustrated in Table I, were performed by adding glucosone-1-C^{14} to a medium containing inactive glucose; the molar ratio of the glucosone to glucose was 0.1. After growth of the streptococcus the HA was isolated and degraded. It will be noted that, although the molar ratio of glucosone to glucose in the medium was 0.1, the ratio of the radioactivity of the glucosamine to that of the glucosone in the medium was only 0.031 (924/29,664) in Experiment 1 and 0.021 (516/25,104) in Experiment 2.

It is apparent from the data in Table I that some of the radioactivity of the glucosone appears not only in the glucosamine portion of the HA, but in other portions of the HA (compare the total activity of glucosamine, 924, to that of HA, 1862) as well as in the acetic and lactic acids of the medium. The relative distribution of activity in these various compounds is similar to that observed previously in experiments with glucose-1-C^{14} and glucose-6-C^{14} (1, 10), except that acetic acid and lactic acid are relatively more highly labeled. The results of this experiment suggest

that, if glucosone is an intermediate in the formation of glucosamine, it must also be assumed to be an intermediate in the formation of other portions of the hyaluronic acid molecule. The localization of the radioactivity of the HA not accounted for by the glucosamine could be readily

TABLE I

Utilization of Glucosone-1-C^{14} in Synthesis of Hyaluronic Acid

Compound	Radioactivity*		Radioactivity* calculated as C-1	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
	c.p.m.	*c.p.m.*	*c.p.m.*	*c.p.m.*
Glucosone in medium	4944	4184	29,664	25,104
HA	133		1,862	
Glucosamine from HA	154	86	924	516
C-6	18		18	
Benzimidazole of medium acetic acid	61	75	488	600
" " " lactic "	44	59	396	531

* All samples were counted as $BaCO_3$ in an internal gas flow counter. Radioactivity is expressed as counts per minute corrected to "infinite thickness."

TABLE II

Utilization of Uniformly Labeled Glucosone in Synthesis of Hyaluronic Acid

Compound	Radioactivity*
	c.p.m.
Glucosone in medium	5210
Glucosamine from HA	178
CO_2 from glucuronic acid	135
Acetate from medium†	148

* All samples were counted as $BaCO_3$ in an internal gas flow counter. Radioactivity is expressed as counts per minute corrected to "infinite thickness."

† Obtained as the benzimidazole derivative. The activity of the acetate carbon = 4 × the activity of the benzimidazole derivative.

established by utilizing uniformly labeled glucosone, since the C-6 of glucuronic acid is readily obtained by hydrolysis of HA by 4 N HCl.

An experiment utilizing uniformly labeled glucosone is illustrated in Table II. The ratio of the radioactivity of the isolated glucosamine to that of the glucosone is 0.034, the results being similar to those in the two experiments in Table I. The C-6 of the glucuronic acid has an activity similar to that of the average activity of the 6 carbon atoms of the glucosamine. Since previous studies have demonstrated that glucose serves as

a precursor of the glucuronic acid portion of the HA molecule in this organism, it seems possible that equal labeling of the glucuronic acid and glucosamine moieties may result from the conversion of glucosone to glucose. This is in keeping with the finding by Becker and Day (4) that glucose isolated from liver glycogen of rats to which labeled glucosone had been administered was as highly labeled as was that obtained from rats receiving an equal amount of labeled glucose.

SUMMARY

The incorporation of C^{14} from both glucosone-1-C^{14} and uniformly labeled glucosone into hyaluronic acid synthesized by a strain of group A hemolytic streptococcus has been studied. Radioactivity was found in the glucosamine and glucuronic acid portions of the molecule, as well as in the acetic and lactic acids produced during growth. Contrary to previous suggestions, no evidence was found to suggest that glucosone is an intermediate in glucosamine synthesis.

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THE ISOLATION AND ACTION OF CRYSTALLINE GLYOXYLIC ACID REDUCTASE FROM TOBACCO LEAVES

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An enzyme which catalyzes the oxidation of reduced pyridine nucleotides by glyoxylate has recently been detected in spinach leaves (1, 2). This enzyme, glyoxylic acid reductase, was purified approximately 10-fold and was found to catalyze Reaction 1. It was further shown that Reaction 1 could

(1) $\underset{\text{Glyoxylate}}{CHO—COO^-} + DPNH^1 + H^+ \rightleftharpoons \underset{\text{Glycolate}}{CH_2OH—COO^-} + DPN^+$

be coupled to the reaction catalyzed by glycolic acid oxidase, a flavoprotein isolated from green leaves (1), which promotes Reaction 2 in the presence

(2) $$\text{Glycolate} + \tfrac{1}{2}O_2 \rightarrow \text{glyoxylate} + H_2O$$

of catalase. The combination of Reactions 1 and 2 provides an effective means of transferring hydrogen from reduced pyridine nucleotides to molecular oxygen, and this system of terminal respiration has been shown to operate in spinach leaf preparations and in model systems (1, 2).

Glyoxylic acid reductase has now been detected in leaves of a number of species, tobacco leaves being an especially rich source. In order to study Reaction 1 in more detail, this enzyme has accordingly been prepared from tobacco leaves and purified until it separated in crystalline form. The properties of the enzyme, the stoichiometry and reversibility of the reaction, the specificity for DPNH, the action of hydroxypyruvate as a substrate, and the effect of inhibitors have also been investigated. Kinetic studies with *p*-chloromercuribenzoate as an inhibitor have suggested that glyoxylate and hydroxypyruvate can combine with the enzyme through the agency of thiol groups present in the protein structure, and that DPNH forms at least two types of enzyme compounds with the protein thiol groups. An unusual stimulatory effect of inorganic anions upon the rate of oxidation of DPNH by hydroxypyruvate has been observed.

[1] The following abbreviations are used: DPN^+ and DPNH for oxidized and reduced diphosphopyridine nucleotide, respectively; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Isolation of Crystalline Enzyme

Assay—The assay is based upon measurement of the rate of oxidation of DPNH in the presence of glyoxylate by observing the decrease in optical density at 340 mμ in the Beckman spectrophotometer at about 25°. To a cell of 1 cm. light path are added 100 μmoles of potassium phosphate buffer, pH 6.4, 49.5 μmoles of sodium glyoxylate, enzyme solution, and water to bring the final volume to 3.0 ml. DPNH, 0.1 to 0.2 μmole, is added at zero time, and readings are taken at 15 second intervals for 1 minute. When necessary, the enzyme is diluted with 0.005 M ethylenediaminetetraacetate (Versene)-0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 per cent gelatin.

The unit is defined as the amount of enzyme that brings about a decrease in optical density of 0.01 per minute at 340 mμ. From 1 to 25 units can be conveniently estimated in the assay procedure. Protein is determined by measuring the light absorption at wave-lengths 260 and 280 mμ (3).

Crude extracts of green leaves usually oxidize DPNH slowly without the addition of glyoxylate. This activity is small compared with the rate in the presence of added glyoxylate, and a correction for the endogenous activity can be made. The low pH, about 5.3, of tobacco leaf extracts destroys the DPNH oxidase activity without affecting the glyoxylic acid reductase; hence no corrections are necessary when tobacco leaves are used

The method of purification of the enzyme from 14 kilos of fresh tobacco leaves is described in a representative experiment summarized in Table I. Mature leaves of *Nicotiana tabacum*, var. Connecticut shade-grown, were picked from plants grown in the field, brought quickly to the laboratory, weighed, and washed with tap water. Each step in the following procedure was controlled by the assay.

Step 1. Preparation of Crude Extract—The leaves were torn up and ground in a Nixtamal mill, and the resulting fine pulp was collected in a container chilled with cracked ice. The green suspension was mixed with sufficient filter paper clippings to form a soft mass which was wrapped in suitable quantities in drill cloth and the juice was expressed in a hydraulic press. The press juice, which is faintly green in color and slightly turbid, was filtered through a thick pad of paper pulp (4) on a Büchner funnel in the cold. The clear yellowish solution which was at pH 5.4 was adjusted to pH 6.3 by the dropwise addition of 2.5 N potassium hydroxide. The specific activity (units per mg. of protein) in various preparations has ranged from about 5 to 11. The temperature was maintained below 5° throughout the rest of the procedure unless otherwise stated.

Step 2. Fractionation with Ammonium Sulfate—Solid ammonium sulfate was added in the proportion 15.4 gm. for each 100 ml. of enzyme solution

to make the concentration 0.22 of saturation.[2] After being stirred for 30 minutes, the suspension was allowed to stand in the cold overnight. Most of the supernatant liquid was siphoned off and collected the next morning, and the remainder of the precipitate was removed by filtration on fluted filter papers and discarded.

To each 100 ml. of supernatant fluid, 12.6 gm. of ammonium sulfate were added to make the solution 0.40 of saturation. The precipitate was collected, washed off the filter papers, and dissolved in one-fifteenth of the original volume of 0.01 M potassium phosphate-0.005 M ethylenediamine-

TABLE I

Isolation of Glyoxylic Acid Reductase

14 kilos of fresh tobacco leaves.

Step No.	Volume	Arbitrary enzyme units per ml.	Total units $\times 10^{-3}$	Protein	Specific activity	Recovery
	ml.			*mg. per ml.*	*units per mg. protein*	*per cent*
1. Clear extract	14,000	136	1900	15.7	8.7	
2. Ammonium sulfate fraction, 0.22–0.40 saturation	1,085	1,720	1870	76.5	22.5	99
3. Supernatant fluid of protamine sulfate ppt	1,320	1,360	1790	41.0	33.2	94
4. Ammonium sulfate fraction, 0.17–0.30 saturation	390	4,000	1560	33.3	120	82
5. Calcium phosphate gel, column chromatography	2,040	390	796	0.317	1230	42
6. Ammonium sulfate fraction, 0–0.40 saturation	13.5	48,000	648	16.4	2930	34
7. Crystallization	12.0	40,700	488	6.53	6220	26
Mother liquor of crystals	22.0	7,090	156	4.66	1520	8

tetraacetate buffer at pH 7.0. If all of the precipitate does not dissolve after being stirred for 15 minutes, the solution should be clarified by centrifugation and the sediment discarded.

Step 3. Purification with Protamine Sulfate—The enzyme solution was dialyzed with stirring for 4 hours against 13 volumes of water. About 1 mg. of protamine sulfate in solution at a concentration of 20 mg. per ml. (adjusted to pH 6 with sodium hydroxide and filtered) was added for every 15 mg. of protein and the solution was stirred for 30 minutes. The precipitate was then removed by centrifugation and discarded.

Step 4. Second Ammonium Sulfate Fractionation—This step was carried

[2] Here and elsewhere it is assumed that a saturated solution of ammonium sulfate is produced by adding 70 gm. of the solid salt per 100 ml.

out as described above, except that the fraction which precipitates between 0.17 and 0.30 of saturation of ammonium sulfate was collected. This precipitate was dissolved in 0.01 M potassium phosphate-0.005 M ethylenediaminetetraacetate, pH 7.0, one-fifth of the volume present at the end of Step 3 being used. The enzyme solution was dialyzed for 4 hours against 35 volumes of water with mechanical rotation of the dialysis sac.

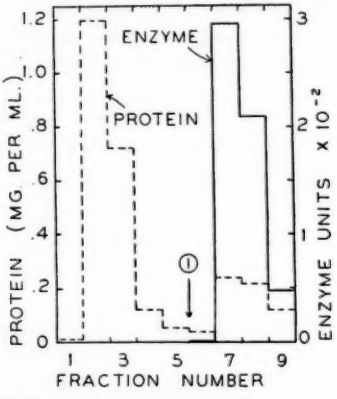

FIG. 1. Calcium phosphate gel chromatography of glyoxylic acid reductase. Column prepared by mixing 110 gm. of dry paper pulp, 2570 ml. of water, and 550 ml. of calcium phosphate gel (33 mg. per ml.) and pouring the suspension into a chromatography cylinder 8.5 cm. in diameter. Column height 16 cm. 275 ml. of a cold dialyzed solution containing 430,000 units of the enzyme of specific activity 55.5 at a stage of purification represented by Step 4, Table I, were forced into the column under pressure of 3 pounds per sq. in. to give a flow rate of one to two fractions per hour. Elution through Fraction 5 was carried out with ammonium sulfate solution containing 0.35 gm. per 100 ml. (weight per volume), fractions of 550 ml. being collected. At Arrow 1, the concentration of ammonium sulfate solution was increased to 1.75 gm. per 100 ml. (weight per volume). Assay for glyoxylic acid reductase resulted as follows: Fraction 6, 0 unit; Fraction 7, 163,000 units, specific activity 1220; Fraction 8, 116,000 units, specific activity 980; Fraction 9, 28,000 units, specific activity 410.

Step 5. Calcium Phosphate Gel Column Chromatography—This technique is based on the postulate that, in situations in which calcium phosphate gel can be used to purify proteins by a batch method, purification should be more efficient with use of column chromatography.

Experience with the batchwise addition of calcium phosphate gel had shown that glyoxylic acid reductase is not adsorbed in the presence of ammonium sulfate at 0.03 of saturation (5). This property of the protein has been exploited to effect extensive purifications with use of columns in which the gel is supported on filter paper pulp. The entire chromatography

step is carried out at room temperature. The results of a typical experiment are presented in Fig. 1. Sober and Peterson (6) have independently reported the use of a similar column technique for the purification of proteins.

Fractions 7 and 8, obtained as shown in Fig. 1, usually contained the enzyme purified from 10- to 30-fold over the previous step and in yields of 50 to 70 per cent. The fractions containing the most active enzyme are combined and made 0.01 M with potassium phosphate-0.005 M ethylenediaminetetraacetate buffer at pH 7.0.

Step 6. Concentration by Ammonium Sulfate Precipitation—Ammonium sulfate was added in the amount necessary to bring the total concentration to 0.40 of saturation (25.9 gm. per 100 ml.). The suspension was allowed to stand for several days, and the precipitate was collected by centrifugation and dissolved in the smallest possible volume of 0.01 M potassium phosphate-0.005 M ethylenediaminetetraacetate buffer at pH 7.0.

Step 7. Crystallization—The enzyme solution was dialyzed with slow stirring at 5–6° against a large volume of 0.01 M potassium phosphate-0.005 M ethylenediaminetetraacetate buffer at pH 7.0, to which ammonium sulfate had been added to 0.12 of saturation (8.40 gm. per 100 ml.). The concentration of ammonium sulfate in the outer solution was slowly raised by adding small amounts of solid salt at intervals of a few hours; so that about 0.17 of saturation was reached in 24 hours. Within 2 or 3 hours after reaching this concentration, a silky sheen could be observed upon stirring the contents of the dialysis sac. Stirring was continued and the concentration of ammonium sulfate was slowly increased to 0.19 of saturation during the next 24 hours in order to obtain more complete crystallization, and equilibration was occasionally continued for another 24 hours.

The enzyme crystallizes in clusters of small needles, about 8 μ in length, with a refractive index close to that of the ammonium sulfate solution. They can be seen best under the phase microscope, especially after being stained with methylene blue (7).

The crystalline glyoxylic acid reductase can be stored frozen for many months or as a suspension in ammonium sulfate in the cold without loss of activity. Recrystallization, however, results in a loss in total activity as well as a decrease in specific activity. In some experiments crystalline preparations with specific activity as high as 8600 have been obtained. This corresponds to the reduction of 4140 moles of glyoxylate per 100,000 gm. of enzyme per minute under the conditions of the assay.

Chemical Balance of Glyoxylic Acid Reductase Reaction

Purified glucose dehydrogenase from ox liver (8) has been used to generate DPNH (Reaction 3) for the reaction catalyzed by glyoxylic acid reduc-

(3) Glucose + DPN^+ + $H_2O \rightleftharpoons$ gluconate + DPNH + H^+ (glucose dehydrogenase)

(1) Glyoxylate + DPNH + $H^+ \rightleftharpoons$ glycolate + DPN^+ (glyoxylic acid reductase)

Sum (3) + (1), glucose + glyoxylate + $H_2O \rightarrow$ gluconate + glycolate

tase (Reaction 1) in experiments designed to test the stoichiometry of the reduction of glyoxylate to glycolate. If the combined reactions are carried out in a Warburg vessel in bicarbonate buffer, 1 mole of carbon dioxide (gluconate) should be liberated per mole of glyoxylate used up and 1 mole of glycolate should be formed. It has been found that, to obtain any evolution of carbon dioxide, the presence of DPN^+ and glucose dehydrogenase is essential. A complete dependence for glyoxylic acid reductase could not be shown, because the glucose dehydrogenase preparations available contained small amounts of glyoxylic acid reductase or lactic dehydrogenase, either of which can function with glyoxylate as a substrate. Glyoxylate disappearance was measured by colorimetric determination of the 2,4-dinitrophenylhydrazone (9), and glycolate formation with use of purified spinach glycolic acid oxidase (1, 10). In other experiments, glycolate has been determined by separating it on paper chromatograms with the solvent system of Denison and Phares (11), followed by a colorimetric estimation of the glycolic acid after elution from the paper (12). Table II shows that, within experimental error, for each mole of carbon dioxide evolved (gluco-

TABLE II

Chemical Balance of Dismutation between Glucose and Glyoxylate

The complete test system in the Warburg vessels contained glyoxylate as indicated, 0.3 μmole of DPN^+, 600 μmoles of glucose, 64 units of glucose dehydrogenase, 150 μmoles of potassium bicarbonate, 41 γ of glyoxylic acid reductase in Experiment 1 and 57 γ in Experiment 2. Final volume, 3.0 ml.; gas phase, 5 per cent carbon dioxide and 95 per cent nitrogen; pH 7.9; temperature, 30°. The reaction was started by tipping in glyoxylate and DPN^+ and continued until carbon dioxide evolution had practically ceased, 9 hours in Experiment 1 and 6 hours in Experiment 2. 200 μmoles of sulfuric acid were tipped in at the end, and aliquots were analyzed for glyoxylate and glycolate as described in the text. Essentially no carbon dioxide was evolved in the absence of DPN^+ or glucose dehydrogenase.

Experiment No.	Contents	Initial glyoxylate	Carbon dioxide evolved	Glyoxylate used up	Glycolate produced
		μmoles	*μmoles*	*μmoles*	*μmoles*
1	Complete	14.5	11.7	10.7	10.7
	Without glyoxylic acid reductase	14.5	3.1	3.7	4.7
2	Complete	11.1	9.0	7.5	7.2
	"	22.2	14.0	15.4	12.8
	Without glyoxylic acid reductase	22.2	2.2	1.3	3.2

nate), 1 mole of glyoxylate is used during the formation of 1 mole of glycolate.

Reversibility of Reduction of Glyoxylate

If high concentrations of the reactants, alkaline pH, and relatively large amounts of enzyme are used, it is possible to demonstrate the oxidation of glycolate by measuring the amount of DPNH formed at 340 mμ (Fig. 2). The addition of a small amount of glyoxylate is sufficient to oxidize com-

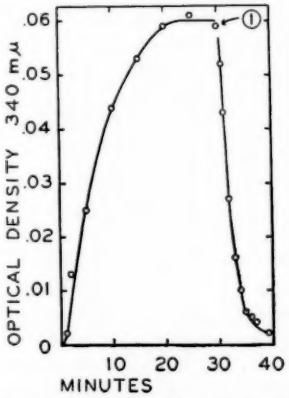

FIG. 2. Reversibility of the glyoxylic acid reductase reaction. The cuvette (l = 1.0 cm.) contained 50 μmoles of sodium pyrophosphate buffer, pH 8.40, 2000 μmoles of potassium glycolate, 1.34 μmoles of DPN^+, and water to make a final volume of 3.0 ml. At zero time, 1 mg. of glyoxylic acid reductase was added. Readings were taken at 25° against a blank cell containing all the components except DPN^+. At Arrow 1, 5.0 μmoles of sodium glyoxylate were added. The pH was measured when equilibrium was attained and at the end of the experiment and found to be unchanged. There was no detectable formation of DPNH in the absence of glycolate.

pletely the DPNH formed by a high concentration of glycolate. The demonstration of the reversal of the reduction of glyoxylate supports the validity of Reaction 1.

The equilibrium constant for the enzymatic reduction of glyoxylate can be determined from experiments in which the amount of DPNH formed from DPN^+ is measured in the presence of a large excess of glycolate.

$$K = \frac{(\text{glycolate})(DPN^+)}{(\text{glyoxylate})(DPNH)(H^+)}$$

Known concentrations of glycolate and DPN^+ are mixed at 25° with glyoxylic acid reductase. The DPNH concentration, as well as that of the

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glyoxylate, is determined at equilibrium from the increase in optical density at 340 mμ. The molar extinction coefficient for DPNH was taken to be 6.22×10^6 sq. cm. × mole^{-1} (13). At the end of the reaction the hydrogen ion activity is determined with a glass electrode.

The determination of K in the range pH 7.82 to 8.40 is presented in Table III. At pH 7.30, the amount of DPNH produced in similar experiments was too small for accurate measurement, and at pH 8.75 the rate of reaction was very slow.

TABLE III

Equilibrium Constant of Reaction Glyoxylate + DPNH + H^+ ⇌ Glycolate + DPN^+

The cuvettes contained the concentrations of glycolate and DPN^+ indicated, 100 μmoles of sodium pyrophosphate buffer, and 1 mg. of glyoxylic acid reductase in a final volume of 3.0 ml. Temperature, 25°. DPNH formation was followed spectrophotometrically at 340 mμ, and the pH was determined after equilibrium had been attained, within 45 minutes in all experiments.

pH	H^+	Glycolate	Initial DPN^+	DPNH	Final DPN^+	K
	moles per l. × 10^8	*mole per l.*	*moles per l.* × 10^4	*moles per l.* × 10^4	*moles per l.* × 10^4	× 10^{-14}
7.82	1.51	1.00	4.47	6.73	4.40	6.43
7.83	1.48	1.00	4.47	6.40	4.41	7.28
7.85	1.41	0.50	8.94	7.30	8.87	5.91
8.00	1.00	0.50	4.47	8.00	4.39	3.44
8.10	0.794	1.00	4.47	10.1	4.37	5.40
8.30	0.501	0.50	4.47	8.50	4.38	5.77
8.40	0.398	0.67	4.47	9.46	4.38	8.20
Mean						6.06 ± 1.51

From the mean value 6.06×10^{14} for K, the standard free energy change was calculated from the relationship $\Delta F^\circ = -RT \ln K$ and was found to be −20,100 calories per mole. If Burton's (14) value of −0.320 volt for the E'_0 at pH 7 for the DPN^+-DPNH system is taken, the oxidation-reduction potential for the reaction glycolate ⇌ glyoxylate + $2H^+$ + $2e$ can be calculated to be −0.087 volt at pH 7 and 25°.

Properties of Enzymatic Reaction

Specificity for DPNH—Crude preparations of tobacco leaf extracts have some activity with TPNH as the hydrogen donor in the reduction of glyoxylate, as do spinach leaf extracts (2). The activity with TPNH is quickly destroyed or removed during the early steps of the purification of the enzyme. There were indications that tobacco leaves contain a different protein specific for TPNH in the reduction of glyoxylate. The inabil-

ity of a large excess of the purified glyoxylic acid reductase to function with TPNH is shown in Fig. 3. Other experiments have shown that the addition of TPNH does not affect the rate of the reaction with DPNH.

Substrate Specificity—Muscle lactic dehydrogenase has been found to be active with a large number of keto acids, glyoxylate being outstanding (15).

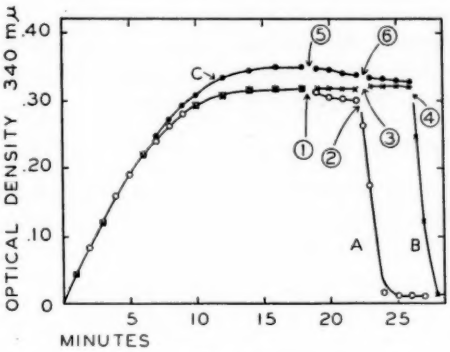

FIG. 3

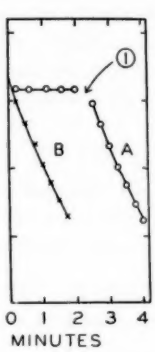

FIG. 4

FIG. 3. Specificity of glyoxylic acid reductase for DPNH. For Curve A, the cuvette (l = 1.0 cm.) contained 100 μmoles of potassium phosphate buffer, pH 6.4, 400 μmoles of glucose, 4.8 units of ox liver glucose dehydrogenase, and water to a final volume of 3.0 ml. 0.15 μmole of DPN^+ was added at zero time. At Arrow 1, 49.5 μmoles of sodium glyoxylate were added; at Arrow 2, 3 γ of glyoxylic acid reductase were added. Curve B, same as Curve A initially. At Arrow 1, no addition; at Arrow 3, 3 γ of glyoxylic acid reductase were added; at Arrow 4, 49.5 μmoles of glyoxylate were added. Curve C, same as Curve A at beginning, except that 0.17 μmole of TPN^+ was used in place of the DPN^+. At Arrow 5, 49.5 μmoles of glyoxylate were added; at Arrow 6, 30 γ of glyoxylic acid reductase were added. Curves A, B, and C were the same during the first 6 minutes.

FIG. 4. Absence of lactic dehydrogenase activity in crystalline glyoxylic acid reductase. For Curve A, the cuvette (l = 1.0 cm.) contained 100 μmoles of potassium phosphate buffer, pH 6.4, 10 μmoles of potassium pyruvate, and 20 γ (112 units) of glyoxylic acid reductase in a final volume of 3.0 ml. At zero time, 0.15 μmole of DPNH was added. At Arrow 1, 12 units of muscle lactic dehydrogenase were added. Curve B, same as Curve A without glyoxylic acid reductase present; lactic dehydrogenase added at zero time.

The purified glyoxylic acid reductase has no activity with pyruvate, nor does the addition of the tobacco leaf enzyme affect the rate of action of muscle lactic dehydrogenase on this substance (Fig. 4). This proves that glyoxylic acid reductase is quite distinct functionally from lactic dehydrogenase. Tests with from 3 to 30 μmoles of pyruvate in the usual assay system, with large amounts of glyoxylic acid reductase, have consistently failed to show any activity with this compound. Similar tests for the ac-

tivity of acetaldehyde have also indicated that the crystalline tobacco leaf enzyme contains no alcohol dehydrogenase.

The virtual absence of glycolic acid oxidase activity in the crystalline glyoxylic acid reductase can be shown. Although crude extracts of tobacco leaves are a rich source of glycolic acid oxidase (10), it was found that 120 γ of the crystalline enzyme had no glycolic acid oxidase activity when tested manometrically in the presence of excess riboflavin phosphate (1). When tested under the conditions of the enzymatic assay, 10 μmoles of α-ketoglutarate and 30 μmoles each of oxalacetate, phenylglyoxylate, or mesoxalate had no activity with 2 γ of glyoxylic acid reductase.

Activity of Glyoxylic Acid Reductase with Hydroxypyruvate

Stafford, Magaldi, and Vennesland (16) have demonstrated the enzymatic reduction of hydroxypyruvate to D-glycerate by preparations from higher plants (Reaction 4). They have shown that this enzymatic activ-

(4) $\underset{\text{Hydroxypyruvate}}{CH_2OH—CO—COO^-} + DPNH + H^+ \rightleftharpoons \underset{\text{D-Glycerate}}{CH_2OH—CHOH—COO^-} + DPN^+$

ity differs from that of muscle lactic dehydrogenase which produces L-glycerate from hydroxypyruvate. Their preparations from green leaves that were active with hydroxypyruvate also failed to catalyze the oxidation of DPNH by pyruvate.

Glyoxylic acid reductase at all stages of purification reduces hydroxypyruvate as indicated in Reaction 4. The assay was identical to that for glyoxylic acid reductase, except that 2 to 3 μmoles of hydroxypyruvate were used in place of glyoxylate. The ratio of the activity with glyoxylate to that with hydroxypyruvate was examined in a number of experiments during the purification procedure. In the example shown in Table I, which is typical in this respect, the ratio of activity with glyoxylate compared to that with hydroxypyruvate was for Step 1, 1.0; Step 2, 2.2; Step 3, 3.1; Step 4, 2.9; Step 5, 2.5; Step 6, 2.7; first crystals, 2.8; crystallized twice, 2.9; crystallized three times, 2.8. Thus beyond Step 2, over a range of about 200-fold purification, the ratio of activities remained constant within experimental error. The relatively higher activity for hydroxypyruvate in the first two steps probably means that there is an enzyme in tobacco leaves that is specific for hydroxypyruvate and which is removed or destroyed during the early stages of purification of the glyoxylic acid reductase. Repeated attempts to change the ratio further by protein fractionation procedures and by heat denaturation have not been successful. As is shown later, the action of *p*-chloromercuribenzoate, an effective inhibitor, appears to be essentially the same when either substrate is used. It may

therefore be concluded for the present that the activity with hydroxypyruvate is associated with the glyoxylic acid reductase protein.

Equilibrium Constant for Reduction of Hydroxypyruvate—The equilibrium constant for the reduction of hydroxypyruvate to D-glycerate has been determined in a manner similar to that shown in Table III, and the data are presented in Table IV.

TABLE IV

Equilibrium Constant of Reaction Hydroxypyruvate + DPNH + H^+ ⇌ D-Glycerate + DPN^+

Each cuvette contained the concentration of D- or DL-glycerate indicated, 100 μmoles of buffer (phosphate, pyrophosphate, or Tris·HCl), the DPN^+ indicated, and 10 to 40 γ of glyoxylic acid reductase in a final volume of 3.0 ml. Temperature, 25°. Equilibrium was reached in all experiments within 20 minutes. The concentrations of DL-glycerate are indicated, but the calculations are based upon the D enantiomorph only.

pH	H^+	Glycerate	Initial DPN^+	DPNH	Final DPN^+	K
	moles per l. × 10^8	*moles per l.* × 10^8	*moles per l.* × 10^5	*moles per l.* × 10^5	*moles per l.* × 10^4	× 10^{-12}
7.0	10.0	16.7 (D)	10.1	0.673	10.0	3.69
7.5	3.16	13.3 (DL)	20.2	1.34	20.1	2.36
7.5	3.16	53.4 "	10.1	1.84	9.90	2.47
8.0	1.00	33.4 "	2.02	1.02	1.92	3.09
8.1	0.794	33.4 "	2.02	1.33	1.89	2.24
8.85	0.141	6.67 (D)	4.03	2.68	3.76	2.49
8.85	0.141	13.3 "	4.03	3.53	3.68	2.79
8.85	0.141	20.0 "	4.03	4.17	3.61	2.95
9.2	0.0631	33.4 (DL)	4.03	5.30	3.50	3.31
Mean						2.82 ± 0.48

$$K = \frac{(\text{D-glycerate})(DPN^+)}{(\text{hydroxypyruvate})(DPNH)(H^+)}$$

The equilibrium constant for the reduction of hydroxypyruvate is 2.82 × 10^{12}, indicating a more readily reversible reaction than the reduction of glyoxylate. The ability of the enzyme to catalyze the oxidation of D-glycerate and the unchanged equilibrium constant when one-half the DL-glycerate concentration present is used in the calculation indicate that D-glycerate is the product of the reduction of hydroxypyruvate as in the system described by Stafford, Magaldi, and Vennesland (16). The standard free energy for the reduction of hydroxypyruvate is −17,000 calories per mole. The oxidation-reduction potential for the reaction D-glycerate ⇌ hydroxypyruvate + $2H^+$ + $2e$ is −0.156 volt at pH 7 and 25°.

Effect of Anions on Reduction of Hydroxypyruvate

A number of anomalies were observed during kinetic studies with use of hydroxypyruvate as substrate. The activity of the enzymatic reaction followed first order kinetics only at low substrate concentrations and at higher substrate levels increased more rapidly than would be predicted. When glyoxylate and hydroxypyruvate were added together and the rate of DPNH oxidation was measured, the initial velocities were always greater than the sum of the activities when each substrate was assayed separately.

TABLE V

Anionic Stimulation of Oxidation of DPNH by Hydroxypyruvate

Each cuvette contained 200 μmoles of Tris·HCl buffer, pH 6.9, 0.2 μmole of DPNH, 2.5 μmoles of hydroxypyruvate, and about 3 γ of crystalline tobacco leaf enzyme in a final volume of 3.0 ml. Temperature, 25°. The initial rate of DPNH oxidation was recorded for four successive 15 second periods, after which 200 μmoles of the salt indicated were added and the rate of DPNH oxidation was again observed in the same experiment.

Salt	Ratio of activity with and without added salt
Potassium phosphate, pH 7.0	1.4
Sodium sulfate	2.2
Potassium sulfate	2.0
Ammonium "	2.2
Sodium chloride	2.5
Potassium chloride	2.4
Ammonium "	2.6
Tris·HCl, pH 6.9	2.3
Potassium bromide	3.0
Sodium nitrate	4.0
Potassium nitrate	4.1
Sodium iodide	5.3
Potassium iodide	5.7

It was also observed that enzyme fractions high in ammonium sulfate when assayed without prior dialysis showed considerably greater activity than before the addition of the ammonium sulfate. These apparent discrepancies were cleared up by the observation that the rate of oxidation of DPNH by hydroxypyruvate is markedly stimulated by added anions, as shown in Table V.

The anionic stimulation of the rate of DPNH oxidation requires both hydroxypyruvate and glyoxylic acid reductase. In experiments in which a large excess of DPNH over the concentration of hydroxypyruvate was present, it could be shown that the addition of anions did not affect the amount of DPNH oxidized but merely the rate of the reaction. Likewise,

the addition of anions did not affect the amount of DPNH formed from DPN^+ and D-glycerate. The effect of anionic stimulation is pH-dependent, as was demonstrated by results obtained when 100 μmoles of potassium phosphate buffer and 300 μmoles of ammonium chloride were used under conditions similar to those described in Table V. The ratio of the rate of DPNH oxidation with added ammonium chloride to that without added anion was, at pH 6.4, 5.5, at pH 7.0, 4.2, and, at pH 7.5, 2.8.

Data on the effect of hydroxypyruvate concentration on the initial rate of DPNH oxidation are presented in Figs. 5 and 6. It should be pointed out that the hydroxypyruvate solution used contained sodium bromide in

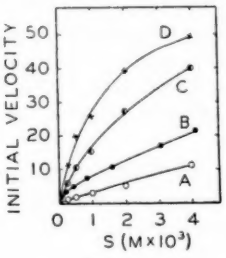

FIG. 5

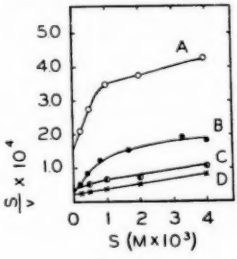

FIG. 6

FIG. 5. Effect of hydroxypyruvate and chloride concentration on initial rate of oxidation of DPNH. Each cuvette contained 100 μmoles of potassium phosphate buffer, pH 6.4, about 2 γ of crystalline glyoxylic acid reductase, 0.2 μmole of DPNH, and hydroxypyruvate as indicated in a final volume of 3.0 ml. Temperature, 25°. Curve A, no ammonium chloride; Curve B, 50 μmoles of ammonium chloride added; Curve C, 100 μmoles of ammonium chloride added; Curve D, 200 μmoles of ammonium chloride added. The initial velocities (v) are expressed in the enzyme units described in the text and the hydroxypyruvate concentration as s.

FIG. 6. Lineweaver-Burk plots of the data in Fig. 5.

a concentration slightly in excess of the concentration of hydroxypyruvate (17). It is clear that, at low hydroxypyruvate concentrations, linear Lineweaver-Burk (18) plots are obtained, but with increasing substrate concentration (increasing bromide concentration as well) the rates are considerably greater than would be expected. Analogous effects brought about by increasing the concentration of fumarate in the fumarase reaction have been reported (19). It is apparent from Fig. 6, in which the slope of the line equals the reciprocal of the maximal velocity, that the effect of added anions is to increase the maximal velocity to a new and higher level which is independent of the salt concentration. Curved Lineweaver-Burk plots have also been observed under certain conditions in studies on fumarase by Alberty and Bock (20). This type of behavior can be explained by any mechanism which can allow for changes of the enzyme whereby more cata

lytic sites become available. It has been suggested (20) that such effects arise from the presence of at least two types of Michaelis-Menten sites on the enzyme which either are different and independent or are identical and can function in pairs.

The stimulatory effect of anions on the rate of oxidation of DPNH by hydroxypyruvate is apparently specific for the substrate hydroxypyruvate and presumably also for the protein. Chloride, in a concentration similar to that used in the experiments in Fig. 5, is slightly inhibitory on the rate of DPNH oxidation by glyoxylate. As mentioned earlier, hydroxypyruvate is an excellent substrate for muscle lactic dehydrogenase, being converted to L-glycerate. The effect of anions on the rate of DPNH oxidation was investigated with use of muscle lactic dehydrogenase and hydroxypyruvate. Under the conditions of the standard enzyme assay, 1.4 units of muscle lactic dehydrogenase were found to be stimulated 1.1-fold by 200 μmoles of sodium chloride, while 1.4 units of glyoxylic acid reductase were stimulated 3.2-fold by the same amount of chloride.

No stimulation in the rate of formation of DPNH by D-glycerate has been observed, but all such tests have been carried out at alkaline pH, and, as has been pointed out, the stimulatory effect decreases with increasing pH.

Inhibitors of Glyoxylic Acid Reductase Reaction

A number of substances have been tested for their ability to inhibit the rate of the enzymatic reduction of glyoxylate. Compounds that showed significant inhibitory effects are listed in Table VI. Some of these are α-keto acids. Other inhibitors, such as cyanide and semicarbazide, probably exert their effect by interacting with glyoxylate. Experience with the coupled glyoxylic acid reductase-glycolic acid oxidase reactions in model systems, such as those described earlier (1, 2), has shown that cyanide is not an effective inhibitor in the presence of an excess of glyoxylate and can even be stimulatory at low concentrations. A number of compounds were found to inhibit the glyoxylic acid reductase reaction 10 per cent or less. These were (at 0.01 M final concentration) mesoxalate, oxalate, malonate, fluoroacetate, fluoride, α-ketoglutarate, formaldehyde, and ethylenediaminetetraacetate; azide (0.001 M) and 2,4-dinitrophenol (0.0001 M) were also not effective. Iodoacetate, 0.01 M, inhibited the reaction 13 per cent after being in contact with the enzyme for 10 minutes and 22 per cent after 20 minutes at 25°. The inhibitor *p*-chloromercuribenzoate, which readily forms mercaptides with the thiol groups of proteins (21), was found to be a powerful inhibitor.

Studies on p-Chloromercuribenzoate Inhibition—Although *p*-chloromercuribenzoate is an effective inhibitor of the reduction of glyoxylate and hydroxypyruvate, the order of addition of the components in the standard

assay system controls the degree of inhibition observed (Fig. 7). If the inhibitor is added to the enzyme before the DPNH and the glyoxylate or hydroxypyruvate, 50 per cent inhibition of the initial rate is obtained at a concentration of from 1.1 to 1.5 × 10^{-7} M *p*-chloromercuribenzoate (Fig. 7, *a*). The inhibition is primarily of the non-competitive type, although larger amounts of glyoxylate are somewhat more effective in diminishing the inhibition than low concentrations. The results of adding glyoxylate or hydroxypyruvate to the enzyme before the *p*-chloromercuribenzoate are shown in Fig. 7, *b*, where 50 per cent inhibition is observed at concentrations of inhibitor of from 3.00 to 20.0 × 10^{-7} M, depending on the concentration

TABLE VI

Inhibitors of Enzymatic Reduction of Glyoxylate

Conditions the same as in the spectrophotometric glyoxylic acid reductase assay. The inhibitor to be tested was added to the solution containing about 3 γ of the enzyme, after which glyoxylate in 0.0165 M final concentration and DPNH were added.

Inhibitor	Inhibition of initial rate
M	*per cent*
Phenylglyoxylate, 0.01	21
Pyruvate, 0.01	56
" 0.001	5
Oxamate, 0.01	31
Oxalacetate, 0.01	22
Arsenite, 0.01	19
2,4-Dichlorophenoxyacetate, 0.01	45
Cyanide, 0.01	57
" 0.001	15
Semicarbazide, 0.01	65

of glyoxylate and hydroxypyruvate present. This behavior can be clearly shown by Lineweaver-Burk plots (18) to be a good example of competitive inhibition, and presumably the glyoxylate and hydroxypyruvate are competing with the inhibitor for the same thiol groups on the enzyme.

As presented in Fig. 7, *c*, the addition of DPNH to the enzyme in advance of the *p*-chloromercuribenzoate affords the greatest protection. The effect of prior addition of DPNH is characterized by curves that show a small inhibition in the range from 10 to 20 × 10^{-7} M inhibitor, and this inhibition is not increased until a concentration of about 75 × 10^{-7} M is reached. However, a second rise in inhibition occurs above this level of concentration of *p*-chloromercuribenzoate. The shape of the curves is very similar for two different concentrations of glyoxylate (Curves A and B) and for one level of hydroxypyruvate (Curve C). The only apparent difference be-

tween the effect of glyoxylate and hydroxypyruvate seems to be in the early portion of the curves where the most easily dissociated enzyme-DPNH compound is most sensitive to the effect of the inhibitor. This may be interpreted as evidence of competition between DPNH and glyoxylate (or

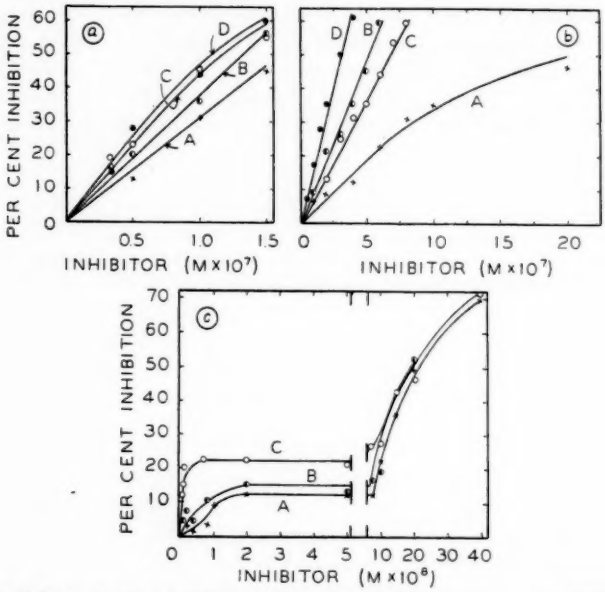

FIG. 7. Effect of prior addition of glyoxylate, hydroxypyruvate, or DPNH upon the inhibition by *p*-chloromercuribenzoate. The experiments were conducted as in the usual enzyme assay, with differences in the order of addition of the components of the assay as follows: (*a*) components added in the order (1) enzyme, (2) inhibitor, (3) glyoxylate or hydroxypyruvate, and (4) DPNH; (*b*) components added in the order (1) enzyme, (2) glyoxylate or hydroxypyruvate, (3) inhibitor, and (4) DPNH; (*c*) components added in the order (1) enzyme, (2) DPNH, (3) inhibitor, and (4) glyoxylate or hydroxypyruvate. Note change in scale of abscissae after the point 5×10^{-4} M. In all figures, Curve A, glyoxylate, 0.0165 M; Curve B, glyoxylate, 0.00413 M; Curve C, hydroxypyruvate, 0.0021 M; Curve D, hydroxypyruvate, 0.000525 M.

hydroxypyruvate) for the same sites (thiol groups) on the protein. These similar effects of *p*-chloromercuribenzoate with glyoxylate and hydroxypyruvate provide further evidence that the protein involved in the reaction is the same.

The great affinity of *p*-chloromercuribenzoate for the enzyme is well illustrated by the demonstration that glutathione (0.001 M), if added to the enzyme after the inhibitor, is ineffective in relieving the inhibition by this

substance. If, on the other hand, the glutathione is added to the enzyme before the *p*-chloromercuribenzoate, almost complete protection is afforded.

Direct Demonstration of Enzyme-p-Chloromercuribenzoate Compound—Although the existence of compounds of enzyme with DPNH has been demonstrated for horse liver alcohol dehydrogenase (22), as well as in other cases, by observations of changes in the spectrum, attempts to observe the formation of a compound between glyoxylic acid reductase and either DPNH or glyoxylate were unsuccessful with the equipment available. Boyer and Segal (23) have shown that the mercaptide which results from the reaction of *p*-chloromercuribenzoate with cysteine or with triosephosphate dehydrogenase has an absorption maximum at 250 mμ. Neilands (24) has applied this characteristic absorption to studies with muscle lactic dehydrogenase and has shown that this enzyme also reacts slowly with *p*-chloromercuribenzoate to form a compound with an absorption maximum at 250 mμ. With use of relatively large amounts of enzyme, it was possible to demonstrate the existence of a glyoxylic acid reductase-*p*-chloromercuribenzoate compound which also has an absorption maximum at 250 mμ. The rate of formation of this compound is illustrated in Curves A and E, Fig. 8. The addition of a concentration of glyoxylate, which would not be expected to protect the enzyme against the concentration of inhibitor used (Fig. 7, *b*), does not affect the amount of enzyme-inhibitor compound formed, as shown in Curve B of Fig. 8, nor does it relieve the inhibition. The prior addition of DPNH, which would be expected to protect the enzyme (Fig. 7, *c*) significantly, diminishes the amount of enzyme-inhibitor compound formed as is illustrated in Curve C, Fig. 8. The reduction in the amount of enzyme-inhibitor under these experimental conditions was accompanied by the preservation of a large part of the enzymatic activity. DPN^+, on the other hand, has no protective effect (Curve D, Fig. 8). These experiments provide direct evidence that DPNH combines with the enzyme through the agency of the same thiol groups which react with *p*-chloromercuribenzoate.

Distribution of Glyoxylic Acid Reductase

The presence of the enzyme has been established in crude extracts of the leaves of spinach, alfalfa, cabbage, and barley. In none of these plants was the specific activity or the total activity on a fresh weight basis as high as that in tobacco leaves. The enzyme has also been detected in pea leaves and seeds and in carrot root (16). Nakada and Weinhouse (25) have suggested that the enzymatic reduction of glyoxylate in mammalian liver is caused by lactic dehydrogenase. All of the purified preparations of ox liver glucose dehydrogenase tested in this laboratory have been found to contain a small amount of glyoxylic acid reductase activity, which would account

for the results shown in Table II and Fig. 3. Under the conditions of the enzymatic assay, the rate of oxidation of DPNH with 49.5 μmoles of glyoxylate is about 5 times that with 10 μmoles of pyruvate. Thus it is still

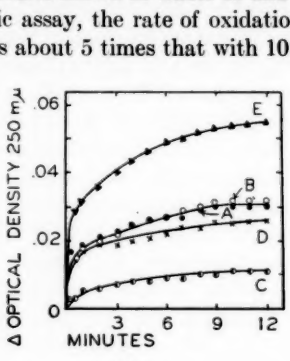

FIG. 8

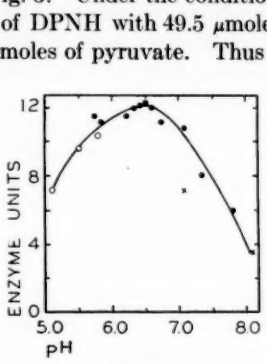

FIG. 9

FIG. 8. Effect of prior addition of glyoxylate, DPNH, and DPN^+ on the formation of an enzyme-*p*-chloromercuribenzoate compound and upon enzymatic activity. The cuvettes (l = 1.0 cm.) contained 100 μmoles of potassium phosphate buffer, pH 6.4, with about 0.33 mg. of crystalline glyoxylic acid reductase in a final volume of 3.0 ml. The blank cells contained the same components without enzyme. The inhibitor, in a final concentration of 100×10^{-7} M, was added to the blank and experimental cells at zero time, and corrections were made for volume changes. Temperature, 25°. Curve A, *p*-chloromercuribenzoate was added to the enzyme at zero time. Curve B, 0.0165 M glyoxylate was added to the enzyme before the *p*-chloromercuribenzoate. Curve C, 0.15 μmole of DPNH was added to the enzyme prior to the addition of *p*-chloromercuribenzoate. Curve D, 0.15 μmole of DPNH and 3.2 μmoles of glyoxylate were added to the enzyme (to oxidize the DPNH completely) before the addition of *p*-chloromercuribenzoate. Curve E, same as Curve A, except 0.66 mg. of enzyme was used. At the final points shown on the curves, when apparent equilibrium had been established, aliquots of 0.03 ml. were assayed for glyoxylic acid reductase activity. An aliquot treated as in the other experiments without addition of *p*-chloromercuribenzoate assayed 14.0 units. In the experiment for Curve C 4.0 units were assayed, and in all of the other experiments less than 0.2 unit was measured.

FIG. 9. Initial rate of glyoxylate reduction as a function of pH. Conditions the same as those in the spectrophotometric assay for glyoxylic acid reductase with about 2 γ of crystalline enzyme. Buffers, ● phosphate, × pyrophosphate, ○ acetate.

possible that mammalian liver also contains a specific glyoxylic acid reductase as well as lactic dehydrogenase.

Kinetics of Enzymatic Reduction of Glyoxylate

Michaelis Constant—With increasing glyoxylate concentration from 0.00207 to 0.033 M, linear Lineweaver-Burk plots were obtained (18). When tested by the standard assay procedure, the Michaelis constant for glyoxylate was found to be 9.1×10^{-3} M. An increase in the concentration

of DPNH in the assay from 3.3×10^{-5} to 13.2×10^{-5} M has no effect on the rate of oxidation of the DPNH; thus the K_m for DPNH is too small to be measured directly. There is no difference in rate between DPNH prepared with dithionate (26) or DPNH made enzymatically with alcohol and alcohol dehydrogenase.

pH Optimum—As presented in Fig. 9, the maximal rate of oxidation of DPNH by glyoxylate was observed between pH 6.3 and 6.6 under the experimental conditions of the assay.

DISCUSSION

Because of the availability of preparations of the enzyme of high specific activity, it has been possible to demonstrate the reversibility of the DPNH-linked reduction of glyoxylate and to determine the equilibrium constant of this reaction. The oxidation-reduction potential for the dehydrogenation of glycolate at 25° (pH 7) is −0.087 volt. The system is therefore one of the most electropositive of the known DPN^+-linked dehydrogenations in which the oxidation of a hydroxyl group to a carbonyl group is involved (27). The DPN^+-linked oxidation of glycolate to glyoxylate by glyoxylic acid reductase is highly endergonic with a $\Delta F°$ of +20,100 calories per mole. Calculations from the oxidation-reduction potentials show that, if the oxidation of glycolate to glyoxylate could be directly coupled to oxygen by an enzyme which is not linked to DPN^+, the reaction would be exergonic with a $\Delta F°$ at pH 7 of about −41,000 calories per mole. It is of interest to note that there is an enzyme in plants, the flavoprotein glycolic acid oxidase, which completely oxidizes glycolate to glyoxylate in the presence of oxygen (1, 10).

Although the activity of glyoxylic acid reductase is similar in some ways to muscle lactic dehydrogenase (15, 24), these enzymes are quite distinct in function. The inhibitory action of pyruvate and other keto acids on the reduction of glyoxylate by the tobacco leaf enzyme indicates that this enzyme can combine with keto acids other than glyoxylate, but it is clear that glyoxylic acid reductase cannot reduce pyruvate to lactate. The available evidence suggests that the ability of the glyoxylic acid reductase to reduce hydroxypyruvate to D-glycerate is associated with the same protein which catalyzes the reduction of glyoxylate. The change in ratio of activity with glyoxylate to hydroxypyruvate during the early stages of purification and the report of wide differences in the ratio of activity with these two substrates in different plants (16) can be attributed to the presence of still another enzyme in plant tissues that is specific for hydroxypyruvate.

Both kinetic and spectrophotometric data have been presented to indicate the existence of enzyme-inhibitor, enzyme-DPNH, and enzyme-substrate compounds in which sulfhydryl groups on the glyoxylic acid reduc-

tase protein are involved. It is difficult to be certain about the order of the affinity of these various compounds for the enzyme, since the velocities of their formation and breakdown cannot be determined from the kinetic studies described. The dependence of the type of inhibition by *p*-chloromercuribenzoate on the sequence of addition of the components to the assay system (Fig. 7, *a* and *b*) may be explained if the rate of cleavage of the enzyme-inhibitor compound by glyoxylate, hydroxypyruvate, and DPNH is a slow reaction and it is assumed that equilibrium had not been attained before the rate measurements were made. In spite of these limitations of the kinetic experiments with *p*-chloromercuribenzoate, it is clear that the protection by DPNH has demonstrated that at least two kinds of enzyme-DPNH compounds are formed, one of which is more readily decomposed by the inhibitor than the other. The protective effect of DPNH is specific, since DPN^+ does not prevent the inhibition by *p*-chloromercuribenzoate nor significantly change the amount of enzyme-inhibitor compound observed spectrophotometrically. The direct spectrophotometric evidence that DPNH prevents formation of the enzyme-inhibitor mercaptide strongly supports the view that the enzyme-DPNH compound is formed by a reaction with thiol groups on the protein and that at least the rate-limiting enzyme-DPNH compound has a dissociation which is of the same order as the enzyme-*p*-chloromercuribenzoate compound.

An anionic stimulation, such as was observed for the rate of oxidation of DPNH by hydroxypyruvate, has not often been reported for enzymatic reactions. However, such effects have been observed with fumarase (28) and with amylase. The stimulatory effect of anions on amylase (29) is in almost exactly the reverse order of effectiveness that has been observed for hydroxypyruvate. The order of decreasing effectiveness for hydroxypyruvate is iodide, nitrate, bromide, chloride, sulfate, and phosphate. The data indicate that additional sites of catalytic attachment must be involved in the reaction of the enzyme with hydroxypyruvate, these additional sites being brought into play by increased concentration of anions.

The possible rôle of glyoxylic acid reductase in the biochemical reactions of green leaves has been discussed earlier (1, 2). James (30) has reviewed the evidence that there is a cyanide-insensitive respiration (probably through a flavoprotein) in plants. From studies with the purified enzymes, both separately and combined in model systems, and with green leaf homogenates, it is clear that the kinetic and thermodynamic properties of the combined glycolic acid oxidase-glyoxylic acid reductase systems are such as to suggest a rôle in respiration. The recent demonstration that isocitrate can be decomposed enzymatically into succinate and glyoxylate by microorganisms (31, 32) illustrates the increasing importance of glyoxylate as a metabolite.

Methods and Preparations

Glyoxylate was prepared from the bisulfite addition compound by the method of Weinhouse and Friedmann (33) and contained sodium chloride. Glyoxylate was determined by the method of Friedemann and Haugen (9) and from the carbon dioxide evolved by oxidation with ceric sulfate (15). Hydroxypyruvate was prepared by the slow hydrolysis of bromopyruvic acid with sodium hydroxide according to the procedure of Sprinson and Chargaff (17) and was determined by the extent of oxidation of DPNH with use of glyoxylic acid reductase or muscle lactic dehydrogenase. Crystalline yeast alcohol dehydrogenase (34) and crystalline rabbit muscle lactic dehydrogenase were kindly supplied by Dr. E. Racker. Glucose dehydrogenase from ox liver (8) was generously provided by Dr. H. Strecker and by Dr. S. Kaufman. Calcium phosphate gel was prepared by the method of Keilin and Hartree (35). Potassium pyruvate was made by the method of Korkes *et al.* (36). Oxalacetic acid was prepared by the method of Heidelberger (37). D-Glycerate was made by hydrolyzing 3-phospho-D-glycerate with the phosphatase present in Taka-Diastase (38), and removing the inorganic phosphate with a Dowex 1 acetate column. The D-glycerate was isolated as the barium salt. Phenylglyoxylic acid was prepared by the oxidation of mandelic acid according to the method of Hurd and McNamee (39). Mesoxalic acid was prepared by the method of Curtiss (40) and was isolated as the sodium salt by the procedure of Clift and Cook (41). Other products were obtained commercially.

SUMMARY

Glyoxylic acid reductase, the enzyme that catalyzes the reaction glyoxylate + DPNH + $H^+ \rightleftarrows$ glycolate + DPN^+, has been isolated from extracts of tobacco leaves in crystalline form. The properties and action of the enzyme have been described. The reversibility of the reaction was demonstrated, and the equilibrium constant was determined to be 6.06×10^{14}. The oxidation-reduction potential for the dehydrogenation of glycolate is −0.087 volt at 25° (pH 7), making it one of the more electropositive of the known DPN^+-linked systems. TPNH does not replace DPNH in the reaction.

The crystalline enzyme also catalyzes the reduction of hydroxypyruvate to D-glycerate but is unable to convert pyruvate to lactate. The equilibrium constant for the reduction of hydroxypyruvate is 2.82×10^{12}, and the oxidation-reduction potential for the D-glycerate-hydroxypyruvate system is −0.156 volt. The rate of the enzymatic oxidation of DPNH by hydroxypyruvate follows unusual kinetics and is greatly stimulated by the addition of anions in the following order of decreasing effectiveness: iodide, nitrate, bromide, chloride, sulfate, and phosphate.

Kinetic and spectrophotometric evidence, with the use of *p*-chloromercuribenzoate as an inhibitor, suggests the existence of enzyme-glyoxylate, enzyme-hydroxypyruvate, enzyme-inhibitor, and at least two kinds of enzyme-DPNH compounds formed through the agency of thiol linkages.

The action of this enzyme is such as to lend further support to the suggestion that glyoxylic acid reductase plays a rôle in the respiration of green leaves.

Grateful acknowledgment is made to Dr. Hubert B. Vickery for his encouragement and for many helpful discussions and to Mr. Laurence S. Nolan for his skillful preparation of the clear leaf extracts. Appreciation is also expressed to the National Science Foundation for a grant which provided part of the financial support for this investigation.

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THE ISOLATION OF PURE HYPOTAURINE FROM THE URINE OF RATS FED CYSTINE*

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A ninhydrin-reacting, sulfur-containing unknown substance was shown to be present, by paper chromatography, in the urine of rats fed a diet supplemented with cystine (1). From the evidence then available the compound was tentatively identified as an incomplete oxidative product of cystamine: either a sulfoxide or a sulfinic derivative. From the pooled urines of a number of rats fed cystine, a small amount of an impure sample of the natural compound was extracted and was shown to contain cystamine disulfoxide by a number of properties (2, 3). From these results the new spot was attributed to the presence of cystamine disulfoxide in the urine of rats. A similar spot, found in the chromatograms of the liver of rats injected with cysteine, was attributed to the same compound (4).

Hypotaurine (2-aminoethanesulfinic acid) synthesized independently by different methods by Awapara (5) and by Cavallini, De Marco, and Mondovì (6) was shown by the latter authors to give, by paper chromatography, the same picture as cystamine disulfoxide, a fact attributable to a dismutation and partial oxidation of cystamine disulfoxide to hypotaurine in the chromatographic run. The suspicion then arose that cystamine disulfoxide contributed only in part to the new spot found in chromatograms from the urine and liver of rats and that hypotaurine could have been present. This was shown to be the case, as far as it concerns the urine, by using ion exchange chromatography. The place of synthetic hypotaurine in the chromatographic pattern of the amino acids in the urine of rats was checked by the method of Stein (7). It was also found that cystamine disulfoxide, under the mild conditions of the ion exchange chromatography, is retained in the column and does not contribute to the hypotaurine peak. By this procedure, a substance having the position and possessing the properties of hypotaurine was shown to be present and was quantitatively estimated in the urine of rats fed cystine (8).

Meanwhile Awapara (5) and Awapara and Wingo (9) found that the injection of cysteine in the rat, in lieu of increasing the amount of alanine, as first reported by Awapara (10), gives rise in the paper chromatogram to a new spot having the same migration characteristics as the spot found by Cavallini *et al.* (1, 4), which they identified as hypotaurine. The identi-

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fication was made by comparing the chromatographic characteristics of the natural with the synthetic compound. In view of the inconclusive evidence given by the experiments of Cavallini *et al.* and Awapara, the isolation of hypotaurine in pure form from the urine of rats fed cystine and its characterization by orthodox chemical and physical methods were deemed necessary. Until now, hypotaurine has been extracted and analyzed only from a liver extract incubated with cysteinesulfinic acid by Bergeret and Chatagner (11).

EXPERIMENTAL

Diet and Collection of Urine—Ten male albino rats were kept in pairs in metabolic cages on the diet previously described (1), containing 6 per cent L-cystine. The urines were collected daily over 2 ml. of 2 N HCl, pooled, and kept in the refrigerator until a total amount of 1000 ml. was obtained. At the end of the collection period the liquid was divided into five portions, decolorized with charcoal, and filtered. The filtrates were placed separately on the anion resin, but the eluates were pooled and treated as one sample in the subsequent steps.

Anion Resin—A bed of 170 ml. of Dowex 2 (80 to 100 mesh) in a column 2.5 cm. in diameter was used as the anion resin column. The regenerative cycles were as follows: 5 N HCl, 300 ml.; CO_2-free water until the effluent was neutral; CO_2-free 1 N NaOH, for 200 ml. after the emergence of the alkaline front; and CO_2-free water until the effluent was neutral.

Cation Resin, Na Form—A bed of 200 ml. of Dowex 50 (80 to 100 mesh) in a column 2.5 cm. in diameter, saturated with NaOH and equilibrated with 0.1 M citric acid, was used as the cation resin, Na form. This column reproduced on a preparative scale the analytical column used by Stein (7) for determination of urinary amino acids. The resolving power, at least for the amino acids eluted with citric acid, is as good as that of the analytical column. With citric acid as eluent, taurine, urea, and hypotaurine are resolved and eluted separately, while the other amino acids are retained on the column (8). The preparative and regenerative cycles were as follows: 5 N HCl, 1000 ml.; water until the effluent was neutral; 0.2 N NaOH, for 200 ml. after emergence of the alkaline front; 0.1 M citric acid, until the effluent was strongly acidic.

Cation Resin, H Form—An amount of Dowex 50 equal to that used for the preparation of the Na form of resin bed was prepared in the H form by the following cycles: 2 N NaOH, 1000 ml.; water until the effluent was neutral; 5 N HCl, 1000 ml.; water, to neutrality of the effluent.

The eluates were collected by a time-regulated fraction collector, the columns being operated at room temperature. Hypotaurine was localized by spotting with a glass rod from every second or fourth tube on a sheet of

Whatman No. 4 paper, drying with warm air, running in phenol, and developing with ninhydrin.

Isolation of Hypotaurine

After the removal of charcoal each of the five portions of urine was passed separately through the anion resin column. The column was washed with 1000 ml. of CO_2-free water, eluted with 1 N acetic acid, and the effluent collected in about 10 ml. portions. Hypotaurine, together with taurine, aspartic acid, glutamic acid, and other unidentified compounds, was eluted in the first 150 ml. after the appearance of the acidic front. The five eluates containing hypotaurine were concentrated to 20 to 30 ml. *in vacuo* with slight warming. The concentrate was transferred to the column of Dowex 50 in the Na form and resolved with 0.1 M citric acid. The effluent was collected in about 4 ml. portions at a rate of 20 ml. per hour. The first 100 ml. of effluent contained taurine and urea, and hypotaurine appeared uncontaminated in the next 200 ml. Citric acid was removed in the next step. The liquid containing hypotaurine was made 1 N with 50 per cent H_2SO_4 and passed through the Dowex 50 column in the H form. The bed was washed with 200 ml. of 1 N H_2SO_4, then with water until the effluent was neutral. The elution was carried out with 1 N ammonia. The bulk of hypotaurine was contained in the first 50 ml. after the ammonia front. Ammonia was removed in a vacuum desiccator over concentrated H_2SO_4 and the solution brought to dryness *in vacuo* with slight warming. The fractionation by Dowex 50 in the Na form and the removal of citric acid were repeated. At the end, a perfectly clear slightly hygroscopic mass of pure hypotaurine was obtained, approximately 150 mg.

Identification of Hypotaurine

The natural hypotaurine has a melting point of 175–177° (micro, uncorrected); a pure sample of synthetic hypotaurine, prepared as described previously (6), melted at 175–177°; previously reported 186–188°;[1] mixed m.p. 174–175°. By paper chromatography the natural compound was indistinguishable from the synthetic one when all the solvents were used. Both the compounds tested on paper gave only one positive spot with the following reagents: ninhydrin, KI + HCl (20 per cent KI in 2 N HCl), $FeCl_3$ (10 per cent in water), and iodoplatinate (12). Microanalyses of the natural compound gave the following:

$C_2H_7NO_2S$.	Calculated.	N 12.83, S 29.37
109.138	Found.	" 12.69, " 29.63

[1] The difference of the present value from that previously reported (6) may be attributable to the use of different apparatus. The reliability of the melting point reported here has been checked by calibrating the micro melting point apparatus (Kofler) with standard compounds.

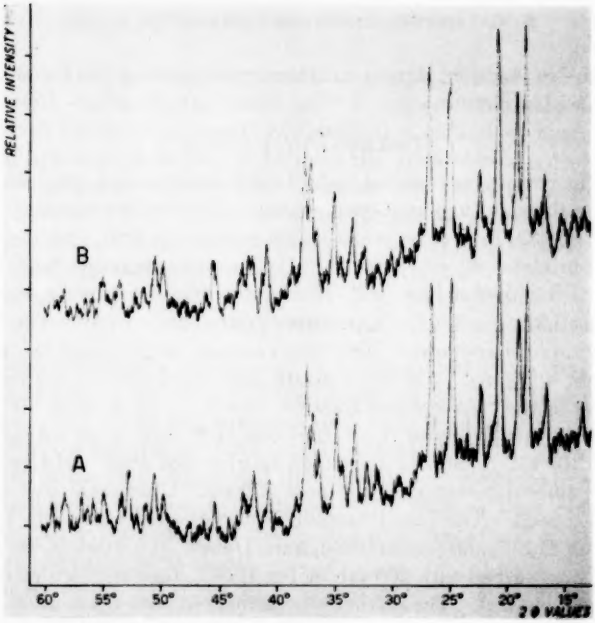

FIG. 1. X-ray diffraction recording of hypotaurine. *A*, synthetic compound; *B*, extracted from the urine of rats fed cystine.

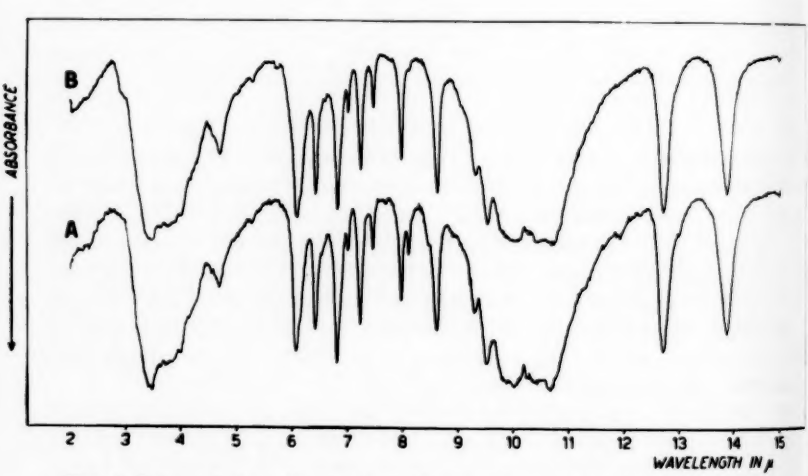

FIG. 2. Infra-red absorption spectra of hypotaurine. *A*, synthetic compound; *B*, extracted from the urine of rats fed cystine. (Suspended in paraffin oil; oil peaks at 3.43, 6.81, and 7.22 μ.)

As further criteria of identity, the x-ray diffraction patterns and infra-red spectra of both the natural and the synthetic compound have been compared. The x-ray powder spectra were obtained by the Brentano parafocusing technique (13) with a General Electric Company automatic x-ray spectrometer. A Perkin-Elmer automatic infra-red spectrophotometer was employed for the infra-red spectra. As shown in Figs. 1 and 2, the Bragg's angles of the diffraction peaks and the wave-lengths of the absorption maxima in the infra-red region are the same for the natural and the synthetic compound.

DISCUSSION

The data presented above are conclusive for the identification of hypotaurine in the urine of rats fed cystine, in agreement with our previous finding (8). Since hypotaurine has been shown to be converted in part to taurine by the living rat (14), its occurrence in the urine corroborates the hypothesis that it may play a rôle in the metabolic reactions leading from cystine to taurine.

Previously hypotaurine has been found to arise from the following compounds: (*a*) cystine *in vivo* (8), (*b*) cysteine *in vivo* (5, 9), (*c*) cysteinesulfinic acid *in vitro* (11) and *in vivo* (15), (*d*) cystamine *in vitro* (16) and *in vivo* (17), (*e*) autoxidation of cystamine and cystamine disulfoxide in alkaline solution (6), (*f*) dismutation of cystamine disulfoxide (6). It is not yet clear what is the route of conversion of cystine to hypotaurine, or whether some one of the above compounds, apart from cystine and cysteine, plays a rôle as an intermediate in this metabolic process. Our first findings on the presence of cystamine disulfoxide in the urine of rats fed cystine are suggestive in pointing to cystamine disulfoxide as one of the intermediates. Nevertheless, the great amount of hypotaurine in the same urine casts some doubt that the disulfoxide we extracted could be an artifact due to the condition of extraction. It is known that a sulfinic acid derivative may be converted to a disulfoxide by heating the acidic solution (18), a procedure which was largely employed in the course of our first extraction of cystamine disulfoxide. Further work is necessary to elucidate this point. Though cystamine is converted to hypotaurine, it is highly unlikely that this compound contributes more than traces to the hypotaurine excreted after feeding cystine. When fed or injected in less than toxic amounts, cystamine is not changed to hypotaurine in the urine of rats (4).

Cysteinesulfinic acid is generally accepted as the most probable precursor of hypotaurine, and it has been unequivocally shown to be decarboxylated to hypotaurine *in vitro* (11, 19). Nevertheless, when fed or injected into rats, cysteinesulfinic acid is converted only in part to hypotaurine, and hypotaurine, though detectable in small amount in the liver (15, 4), does

not appear in the urine (4, 20), as is the case when cystine and cysteine are fed or injected in comparative amounts. This fact necessitates caution in concluding that cysteinesulfinic acid is the main intermediate between cystine and hypotaurine.

SUMMARY

Hypotaurine has been extracted in pure form from the urine of rats fed a diet supplemented with cystine. The natural compound is identical with synthetic hypotaurine, as shown by comparison of the infra-red absorption spectra, x-ray diffraction patterns, and other properties.

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PATHWAYS OF GLYCINE CATABOLISM IN RAT LIVER*

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The most likely path for the oxidation of glycine carbons to CO_2 is generally considered to involve their successive passage through serine, pyruvate, acetyl coenzyme A, and the citric acid cycle (1–4). Establishment of this route as a means of glycine oxidation rests on the observations that glycine and serine are converted to glycogen ((5–8); (1) p. 77), that glycine and serine are interconvertible (8–10), and that serine may be converted to pyruvate (11, 12). Recently, two other routes of glycine oxidation became apparent; one involves successive transformations to glyoxylate, formate, and CO_2 (13), and another is evident in the succinate-glycine cycle discovered by Shemin (14, 15). These pathways are outlined in Diagram 1. In the present report experiments are described bearing on the quantitative significance of the pathway via serine and pyruvate in relation to that via glyoxylate and formate in rat liver, with C^{14}-labeled glycine and related substances.

Methods

To appraise the quantitative significance of the serine-pyruvate pathway, the most direct procedure appeared to be the determination of the extent to which glycine carbon is converted to intermediates. The most convenient means at our disposal for this purpose was the metabolic "trapping" technique. In this type of experiment a suspected intermediary metabolite on a pathway to CO_2 is incubated under oxygen with a tissue preparation containing a C^{14}-labeled substrate. The intermediary participation of the metabolite is established by a significant uptake of C^{14} therein and a marked lowering of the conversion of the labeled carbon to CO_2. Rat liver was chosen for this study because it is one of the few rat tissues which oxidize both glycine carbons rapidly (13), and it has the convenient char-

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† This work is taken in part from a thesis presented by Henry I. Nakada to the Graduate Council of Temple University in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Present address, Scripps Metabolic Clinic, La Jolla, California.

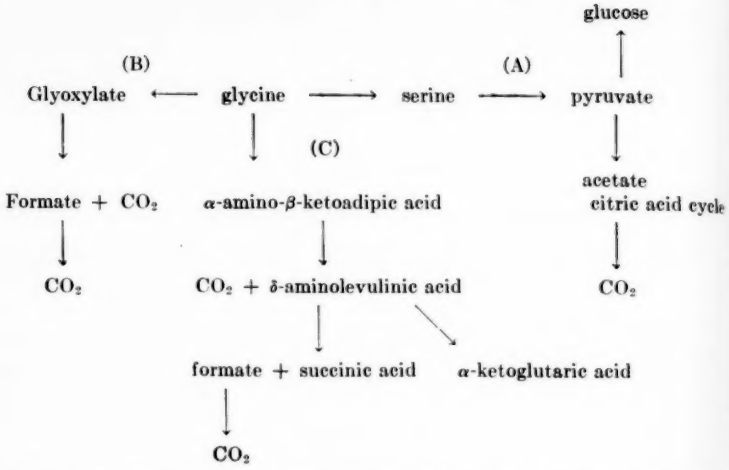

DIAGRAM 1. Glycine catabolism: Pathway A, via serine, pyruvate, and the citric acid cycle; Pathway B, via glyoxylate and formate; Pathway C, via the glycine-succinate cycle.

acteristic of "trapping" metabolic acetyl groups in the form of acetoacetate, which can be isolated readily as acetone in the form of a mercury complex (16). Since acetate is a key intermediate of pyruvate oxidation, the extent of incorporation of glycine carbon into acetoacetate would indicate the extent of pyruvate formation from glycine. To interpret the results of such experiments in their proper perspective, it was considered desirable to include labeled serine and to compare acetoacetate production from these substances with that from a known pyruvate precursor. Lactate-3-C^{14} was chosen for this purpose. By using liver slices it is unnecessary to add a "trapping" agent for acetoacetate, since it accumulates spontaneously under the conditions employed. However, to increase the yield of acetoacetate for ease in isolation, butyrate was added as a cosubstrate. Preliminary orienting experiments revealed that this did not affect the oxidation of glycine or the other isotopic substrates tested. At the same time, the conversion of glycine to serine was measured by incorporation of carbon from the former into an added pool of the latter; formate production was measured from each of the labeled substrates, both in the presence and in the absence of a formate pool, and CO_2 was recovered as usual by trapping with alkali in the center well.

Experiments with slices were conducted by the Warburg technique essentially as described previously (17), with livers from normal, adult rats from our stock colony. Details of each experiment are given in Tables

I to III. At the end of the incubation period, the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 2 per cent, and aliquots of the supernatant solution were used for the following determinations.

Acetoacetate—The deproteinized solution was made basic, was steam-distilled until 100 ml. of distillate were collected, and acetone was precipitated as the mercuric sulfate complex (17).

Formate—The residue from the acetone distillation was acidified with dilute H_2SO_4 and 0.5 mmole of carrier formate was added. This solution was steam-distilled until 600 ml. were collected, and the formic acid oxidized with mercuric sulfate, as previously described (18).

Isolation of Serine—Carrier glycine and serine were added to the residue from the formate distillation. Sufficient $BaCl_2$ was added to remove sulfate ions and, after heating with a small amount of decolorizing charcoal, the solution was centrifuged. The precipitate of $BaSO_4$ and charcoal was washed by resuspension and centrifugation, the combined supernatant fluids were evaporated to a small volume, again decolorized with charcoal, and filtered. The filtrate was transferred to a Dowex 50 column and chromatographed by the method of Hirs, Moore, and Stein (19). Paper chromatography was used to identify and check the purity of the isolated serine.

Degradation of Serine—A portion of the isolated serine was oxidized to CO_2 by the persulfate wet oxidation method, and the β-carbon was obtained from the remainder by periodate oxidation and isolation of the formaldehyde as formaldemethane. The activity of the α-carbon was calculated as total activity minus the activity of the β-carbon, as previous experiments indicated that only negligible amounts of radiocarbon from the α-carbon of glycine appear in the serine carboxyl carbon.

Calculations—The conversions of labeled substrate carbon to the products in question are given in microatoms, according to the formula, the microatoms converted equal the specific activity of the product times the microatoms of the product carbon divided by the specific activity of labeled substrate carbon.

Specific activities were obtained by counting as $BaCO_3$ in "infinitely thick" layers 7.5 sq. cm. in area under a mica window tube.

The labeled compounds were obtained from commercial sources, except glyoxylic acid-1,2-C^{14}, which was prepared as described previously (20). All isotopic carbon was obtained on allocation by the United States Atomic Energy Commission.

Results

Acetoacetate Formation—The results of these procedures are summarized in Table I in which are given data of a single experiment typical of many

such conducted under similar or only slightly different conditions. Glycine carbon 2 was readily oxidized to CO_2, but only a small proportion was converted to acetoacetate. Carbon 3 of serine was also rapidly oxidized to CO_2, but, as with glycine carbon 2, relatively little was incorporated in acetoacetate. On the other hand, lactate carbon 3 was not only oxidized readily but was also incorporated to a marked extent in acetoacetate. In view of the ready incorporation of lactate carbon into acetoacetate, it would appear reasonable to conclude that the carbon of other substances whose metabolism proceeds via pyruvate and acetate will be incorporated

TABLE I

Formation of Labeled Products from C^{14}-Labeled Glycine, Serine, and Lactate

The values are given in microatoms of the labeled substrate carbon incorporated in the product in question per hour per gm. of dry tissue. Each large Warburg vessel contained 3 gm., fresh weight, of pooled liver slices suspended in a total volume of 30 ml. of Krebs-Ringer-phosphate solution, containing substrates and sodium butyrate in final concentration of 0.01 M. Experiments were conducted for 2 hours at 37° with oxygen in the gas phase.

Substrates	Products					
	Respiratory CO_2	Acetoacetate	Formate	Serine		
				Total	β	α
Glycine-2-C^{14}	13.6	0.9	1.0	16.8	8.4	8.4
" + formate	5.9	1.9	11.0	23.0	9.3	13.7
" + serine	7.3	1.9	1.2	58.2	14.0	44.2
DL-Serine-3-C^{14}	47.3	2.3	2.1			
" + formate	23.0	3.0	22.1			
DL-Lactate-3-C^{14}	30.4	60.4	0.8	2.0*		

* Not degraded.

into acetoacetate. On this basis, the lack of appreciable incorporation of glycine carbon 2 or serine carbon 3 into acetoacetate may be taken to indicate that their oxidative metabolism is not directed into acetate to a large extent.

Formate Formation—In those experiments in which glycine-2-C^{14} and serine-3-C^{14} were separately incubated with liver slices, only very small amounts of C^{14} were recovered as formate. Inasmuch as formate is itself readily oxidized by rat liver (13), the low level of C^{14} incorporation may have been due to removal of formate by oxidation rather than to its nonformation. That production of formate is indeed high from both of these positions is indicated from the results of those experiments in which a pool of formate was present to "trap" metabolic formate. In each instance,

large amounts of labeled carbon were "trapped," the difference between the supplemented and unsupplemented vessels being about 11-fold. It is also interesting to note that, in the flasks supplemented with non-labeled formate, CO_2 production from the labeled carbon was drastically curtailed, from 47 to 23 μatoms with serine-3-C^{14} and from 13.6 to 5.9 μatoms with glycine-2-C^{14}. Though interpretation of these results is not entirely unequivocal, the supposition seems warranted that formic acid is a major intermediate on the pathway of oxidation of these carbon atoms to CO_2.

Glycine-Serine Interconversion—It is clear from data on serine production that the inability of glycine to be converted to acetate is not due to lack of conversion to serine. In confirmation of the findings of others (8–10), this was found to be extraordinarily rapid. Even without a serine "trap," considerable serine was formed from glycine, and this was increased somewhat when formate was also present. However, in the presence of a serine pool, incorporation of glycine carbon 2 in serine was increased 3-fold.

The C^{14} distribution in serine obtained from these experiments is of interest in suggesting possible mechanisms for this interconversion. In the serine isolated from the vessel containing no added serine, the α- and β-carbons had equal activities. Thus, essentially the sole source of the C_1 precursor of the serine β-carbon is the glycine α-carbon, and the main route of formation must involve a prior transformation of glycine α-carbon to C_1 followed by a condensation, C_1 + glycine $\rightarrow$ serine. In the presence of formate, the serine was similarly labeled; here the somewhat greater activity of the α-carbon indicates that the added formate was contributing to some extent to the serine β position, but, again, most of the serine β-carbon activity came from glycine carbon 2. However, when a serine pool was present, incorporation of glycine carbon into the α-carbon of serine was over 3 times that in the β-carbon. These findings indicate that serine carbon is itself a more efficient precursor of a C_1 unit for condensation with glycine to yield a new serine molecule than is either formate or glycine carbon. These findings point to a rapid equilibrium of the following nature, serine $\rightleftarrows$ C_1 + glycine, and the rapid incorporation of radioactivity into serine can be attributed to the feeding of labeled glycine into the system.

In Table II there are given results of another type of isotope tracer experiment conducted in a whole homogenate of rat liver, which by themselves are equivocal but which clearly supplement the more decisive experiments already described. Oxidation of the labeled glycine α-carbon is lowered by non-isotopic serine, as might be expected in view of their rapid interconversion, but it was almost entirely abolished by addition of glyoxylate in either the presence or absence of serine. Oxidation of the labeled serine β-carbon was also lowered in the presence of glyoxylate. On the

other hand, neither serine nor glycine lowered the oxidation of labeled glyoxylate. In view of the apparent sluggishness of the serine-pyruvate-acetate pathway of glycine oxidation, as seen in Table I, a reasonable interpretation of these data is that glyoxylate is an intermediate of the oxidation of glycine, but that neither glycine nor serine is a major intermediate in the oxidation of glyoxylate. However, since glyoxylic acid is also an inhibitor of respiration, as seen from its effect on oxygen consumption, the possibility still exists that its action in lowering the incorporation of glycine and serine carbon into CO_2 might be due to inhibition of respiration.

TABLE II

Use of "Trapping" Compounds to Study Pathway of Glycine Oxidation

The rat liver homogenate in this study was prepared by the Potter-Elvehjem technique by using isotonic KCl-phosphate buffer, pH 7.4, as the homogenizing and incubating medium. 330 mg. of tissue were suspended in 3 ml. of total volume, and incubated for 1 hour at 37° with air in the gas phase. All substrate concentrations were 0.01 M. The values are given in microatoms of labeled carbon converted to CO_2 per gm. of tissue (dry weight).

Radioactive substrate	Normal substrates	O_2 uptake	Respiratory CO_2
		µl.	*µatoms C*
None	None	289	
Glycine-2-C^{14}	"	359	29.0
"	Serine	344	13.7
"	Glyoxylate	226	3.5
"	" + serine	226	4.4
Serine-3-C^{14}	None	292	26.0
"	Glycine + glyoxylate	229	12.8
Glyoxylate-1,2-C^{14}	None	129	36.4
"	Glycine + serine	222	38.2

Decisive evidence of the participation of glyoxylate in glycine oxidation was provided in a previously described experiment (13) in which, in a washed liver particulate preparation, large amounts of labeled glycine α-carbon were trapped in glyoxylate. These findings have been confirmed and extended by similar experiments in whole homogenates of rat liver in Table III.

Glyoxylate was isolated from the filtrate after trichloroacetic acid treatment by addition of an excess of 2,4-dinitrophenylhydrazine dissolved in 2 N HCl. The hydrazone was taken up in ethyl ether, then brought into the aqueous phase by extraction with dilute sodium carbonate solution. The aqueous layer was reacidified, extracted with ethyl acetate, the extract evaporated to dryness, and the crystalline residue recrystallized to a constant specific activity with 50 per cent ethanol.

It may be seen in Experiment 1 that the presence of unlabeled glyoxylate lowered the oxidation of glycine carbon 2 from 11 μatoms to approximately 1; at the same time, appreciable labeled carbon appeared in the glyoxylic acid 2,4-dinitrophenylhydrazone. Indeed, the observed incorporation in glyoxylate is to be regarded as minimal, since probably more than half of the hydrazone was lost during its purification.

The possibility that glycine follows the oxidative pathway via glyoxylate only at unphysiologically high concentrations was tested by measuring incorporation into glyoxylate, and into CO_2 of glycine carbon 2 at four different glycine levels ranging from 0.001 to 0.02 M. It was reasoned that

TABLE III

"Trapping" of Glycine Carbon in Glyoxylate during Incubation with Rat Liver Homogenate

In Experiment 1, incubation was conducted for 2 hours at 37° with air in the gas phase. Each vessel contained 3 gm. of whole homogenate suspended in 30 ml. of an isotonic KCl-phosphate buffer solution, 0.02 M in glycine-2-C^{14}, and 0.01 M in glyoxylate, when present. The values are given in microatoms of glycine α-carbon converted. Experiment 2 was similar, except for the variation in glycine-2-C^{14} concentration and a 1 hour incubation period.

Experiment No.	Additions	O_2 uptake	Respiratory CO_2	Glyoxylate
		μl.	*μatoms C*	*μatoms C*
1	Glycine-2-C^{14}	324, 322	11.6, 11.2	
	" + glyoxylate	128, 125	1.4, 1.3	3.31, 4.66
2	" 0.001 M + glyoxylate	78.1	0.09	0.30
	" 0.005 " + "	85.5	0.32	1.36
	" 0.01 " + "	75.8	0.66	4.34
	" 0.02 " + "	75.8	1.14	3.74

the relative amounts incorporated into glyoxylate and oxidized to CO_2 would vary widely if different catabolic routes are followed at different glycine concentrations. The results given in Experiment 2, Table III, indicate a rather close parallelism between the two processes. Increasing the concentration of glycine from 0.001 M to 0.02 M, a 20-fold change, resulted in a 13-fold increase in glycine carbon oxidized to CO_2 and a 12-fold increase in its conversion to glyoxylate. Thus it does not appear that the relative participation of glyoxylate in glycine oxidation varies with changing glycine levels.

DISCUSSION

Our findings indicate that in rat liver neither serine nor glycine is readily convertible to acetoacetate. Inasmuch as lactate, pyruvate, and acetate

are all ketogenic in rat liver, the assumption seems warranted that serine is not readily converted to acetoacetate, and hence, in this tissue, Pathway A in Diagram 1 is not of major significance in the metabolism of glycine. This finding is in accord with the general lack of reactivity of serine toward tissue transaminase (21) and to L-amino acid oxidases ((1) p. 74). Though Chargaff and Sprinson (11) showed that serine can be converted to pyruvate in extracts of mammalian liver, their data do not give an idea of the rate of this process in the intact liver cell.

The apparent non-participation of pyruvate in the metabolism of glycine and serine appears at first glance to be incompatible with the known glycogenic activity of both amino acids (5–8). The possibility exists, however, that glycogen formation may occur via pathways not involving pyruvic acid.[1] It is also possible that glycine, in its catabolism via Pathway B of Diagram 1, can yield glycolaldehyde, which might be incorporated in sugar by the transaldolase and transketolase reactions described by Horecker *et al.* (23) and Racker (24), since it is now apparent that the diose unit transferred in these reactions is closely related chemically to glycolaldehyde.

Another possibility exists that glycine and serine exert only indirect glycogenic activity by stimulating glycogen formation from other substances rather than supplying their own carbon for this process. The isotope tracer data of Olsen *et al.* (7) and of Barnet and Wick (6) show that a relatively small proportion of the glycogen synthesized in response to glycine feeding is derived from the ingested glycine. The observation that the peak of glycogen synthesis occurs 16 hours after glycine feeding (5) and the finding that the glycine α- and β-carbons are incorporated very unequally (6) provide further evidence that glycine carbon may not be directly converted to glycogen by Pathway A of Diagram 1.

Our data shed no light as yet on the possible participation of the succinate-glycine cycle of Shemin (14, 15) in the oxidation of glycine by rat liver. This process yields formate from the glycine α-carbon, and possibly some or all of the formate formed in our experiments could have arisen in this manner. On the other hand our results also demonstrate the formation of glyoxylate, which is known to yield formate (13), and, since it is probable that glyoxylate arises directly from glycine by transamination (25), it seems likely that Pathway B of Diagram 1 is a route of glycine catabolism in rat liver.

It should be pointed out that these conclusions are not yet applicable to the whole animal. As with other metabolites, the extent of participation

[1] Sallach (22) has recently pointed out how serine may be formed from carbohydrate via β-hydroxypyruvic acid. The extent of participation of this substance in glycogen formation from serine remains to be investigated.

of alternative pathways of glycine oxidation in the intact organism and the factors influencing these processes remain problems of future exploration.

SUMMARY

Glycine-2-C^{14}, serine-3-C^{14}, and lactate-3-C^{14} were incubated in oxygen with liver slices and their conversion to various substances was measured by the "trapping" technique. All three were converted to CO_2, whereas only lactate yielded much carbon for acetoacetate formation. Glycine and serine were readily interconvertible, and both yielded much labeled carbon for formate production. The presence of unlabeled glyoxylate greatly lowered the incorporation of glycine and serine carbon into CO_2, but neither serine nor glycine lowered the oxidation of labeled glyoxylate carbon. In homogenates of rat liver, glycine is readily converted to glyoxylic acid.

These findings indicate that a pathway via serine, pyruvate, and acetyl coenzyme A is not a major one for glycine oxidation in rat liver, the block apparently being in the conversion of serine to pyruvate. In contrast, glyoxylate and formate are major intermediates of glycine oxidation in this organ.

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REDUCTION OF CYTOCHROME OXIDASE WITH FERROCYTOCHROME *c**

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Several investigators have attempted without success to demonstrate the reduction of oxidized cytochrome oxidase by ferrocytochrome *c*. Keilin and Hartree (4) employed cytochrome *c* reduced with platinum black and hydrogen, but were unable to detect the appearance of reduced cytochrome oxidase under anaerobic conditions. Their experiments were carried out in modified Thunberg tubes which were evacuated, washed with purified nitrogen, and finally shaken in order to complete the removal of oxygen by the chromous chloride solution placed in a rotating side bulb.

In early experiments (5) we also evacuated our tubes and repeatedly filled them with nitrogen. Although the cytochrome oxidase was partially reduced, the results were not free of criticism (6), since the cytochrome *c* was completely oxidized. However, when special spectrophotometer cells (7) were used which permitted the nitrogen to be bubbled through the solution of cytochrome oxidase, the oxidation of the cytochrome *c* was only partial (8). It is also possible to demonstrate the reduction of cytochrome oxidase by ferrocytochrome *c* in air if sodium cyanide is present (9).

In the experiments to be reported here the use of the special spectrophotometer cell has been improved so that the system under study consists only of ferrocytochrome *c* and oxidized cytochrome oxidase and is not complicated by the presence of traces of oxygen. Thus, it is possible to calculate equilibrium constants, free energy changes, oxidation-reduction potentials, and extinction coefficients.

EXPERIMENTAL

Cytochrome oxidase (termed the Preparation 2-3) was prepared as described previously (10, 3) by the addition of 2 per cent and then 3 per cent of sodium deoxycholate to a preparation of heart muscle particles.

* A preliminary report was presented at the meeting of the American Society of Biological Chemists, Atlantic City, April, 1954 (1). We have been unable to separate cytochrome oxidase into two components, either by ultracentrifugation (2) or by the serial addition of sodium deoxycholate (3). For the present, therefore, it is assumed that only one enzyme acts between cytochrome *c* and molecular oxygen. This component is termed cytochrome oxidase, and to it is assigned all of the increase in absorption at 605 mμ which accompanies reduction.

The lyophilized preparation was dissolved when needed so that each ml. contained the solids originally present in 1 ml. of the non-lyophilized preparation; *i.e.*, "1 ml. equivalent" (3) was dissolved in 1 ml. of distilled water.

Cytochrome c was obtained from Wyeth, Inc.[1] It had been prepared by Tint and Reiss from horse heart and was reported by them to be electrophoretically pure (11, 12). It was stored at 4° in solution (9.7 mg. per ml.) and contained 0.01 per cent of sodium ethyl mercurithiosalicylate as a preservative. Aliquots of the concentrated preparation were diluted with varying amounts of water and with a constant amount (1 ml.) of 0.1 M $Na_2HPO_4 \cdot KH_2PO_4$ buffer, pH 7.4, to obtain solutions containing from 0.097 to 0.97 mg. of cytochrome *c* per 0.5 ml. These solutions were reduced with Linde hydrogen for 15 minutes with platinum black as a catalyst. The platinum black was removed by filtration through Whatman No. 5 filter paper. In each instance the filter paper adsorbed some of the cytochrome *c*. The final concentration of the ferrocytochrome *c* and of the total cytochrome *c* was determined on aliquots of the filtrate.

The *reduction of cytochrome oxidase by ferrocytochrome c* was accomplished at 25° in a modified Thunberg tube (7) adapted for use with the Beckman spectrophotometer. 1 ml. of the cytochrome oxidase solution, 1 ml. of 0.1 M $Na_2HPO_4 \cdot KH_2PO_4$, pH 7.4, and 0.5 ml. of distilled water were added to the body of the tube, while 0.5 ml. of the ferrocytochrome *c* solution was pipetted into the bulb. It was discovered in preliminary experiments that reproducible results could be obtained only if the oxygen-free nitrogen[2] was also passed through the solution in the bulb. Therefore, a 4 inch No. 16 gage hypodermic needle was inserted into the bulb through the venting tube. Nitrogen was passed simultaneously through the solutions in the bulb and in the body for 15 minutes without interfering with the venting action of the tube leading from the bulb. At the end of the 15 minutes, the hypodermic needle was removed from the bulb, while nitrogen was still bubbling through the body. The two stop-cocks were then closed simultaneously. The contents were mixed and optical density readings were taken at 550 mμ and at 5 mμ intervals from 575 to 625 mμ.

Total Cytochrome c Added—Two 0.5 ml. aliquots of each solution of ferrocytochrome *c* were diluted to 3.0 ml. with 0.1 M phosphate buffer, pH 7.4, and water. They were analyzed spectrophotometrically after reduction of the one with sodium dithionite and oxidation of the other with potassium ferricyanide. The formula employed was ferrocytochrome $c = (D_{red.} - D_{ox.}) \times 3 \times 1.227 \times 10^7/(\epsilon$ at 550 mμ reduced $- \epsilon$ at 550

[1] Through the courtesy of Dr. H. Tint.

[2] Matheson prepurified nitrogen passed through two ordinary bubblers containing VO in solution, two Fisher-Milligan gas washing bottles containing VO in solution, and one ordinary bubbler containing distilled H_2O.

TABLE I

Reduction of Cytochrome Oxidase and Oxidation of Ferrocytochrome c

Experiment No.	Total cytochrome c added (1)	Ferrocytochrome c added (2)	Ferrocytochrome c remaining (3)	Ferricytochrome c remaining (4)	Ratio, (4)/(3) (5)	Ferrocytochrome c oxidized, (2)–(3) (6)	Fraction of cytochrome oxidase reduced (7)	Fraction of oxidized cytochrome oxidase remaining (8)	Ratio, (7)/(8) (9)	Ratio, (7)/(6) (10)	K (11)	E'_0 (12)	$-\Delta F$ (13)	ΔD at 605 mμ (14)	$\Delta\epsilon^{1\text{ cm.}}_{M\text{ per l.}}$ at 605 mμ (15)
	mg.	*mg.*	*mg.*	*mg.*		*mg.*						*volt*	*calories per electron*		*10^5 sq. cm. per gm. atom Fe*
1	0.935	0.692	0.463	0.472	1.0	0.229	0.62	0.38	1.63	2.7	1.66	0.267	302	0.041	0.066
2	0.433	0.348	0.153	0.280	1.8	0.195	0.62	0.38	1.63	3.2	2.98	0.282	650	0.041	0.078
3	0.251	0.215	0.043	0.208	4.8	0.172	0.47	0.53	0.89	2.7	4.38	0.291	875	0.031	0.067
4	0.163	0.113	0.013	0.150	11.5	0.100	0.35	0.65	0.54	3.5	6.22	0.301	1090	0.023	0.085
5	0.117	0.087	0.023	0.094	(4.1)	0.064	0.24	0.76	0.32	3.8				0.016	0.093
6	0.055	0.023	0	0.055		0.023	0.06	0.94	0.06	2.6				0.004	0.064
Average											3.81	0.285	729		0.076

mμ oxidized)l, where $D_{red.}$ = the density of the reduced sample, $D_{ox.}$ = the density of the oxidized sample, 1.227×10^7 = the mg. molecular weight of cytochrome *c*, ϵ at 550 mμ reduced = 0.278×10^8 sq. cm. per mole, ϵ at 550 mμ oxidized = 0.090×10^8 sq. cm. per mole, and $l = 1$ cm. The total cytochrome *c* equals the calculated ferrocytochrome *c* when all of the enzyme is completely reduced. The total cytochrome *c* added was somewhat less than the cytochrome *c* originally contained in each 0.5 ml., because the filter paper adsorbed some of the cytochrome *c*. This adsorption was most noticeable in Experiment 6, in which a solution containing 0.097 mg. per 0.5 ml. was prepared and only 0.055 mg. per 0.5 ml. remained to be added (Table I).

Ferrocytochrome c Added—A 0.5 ml. aliquot of each ferrocytochrome *c* solution was diluted to 3.0 ml. with buffer and water and analyzed as above for ferrocytochrome *c*. It was not possible to obtain complete reduction of the cytochrome *c* under the conditions employed. The greatest reduction was 86 per cent (in Experiment 3).

Ferrocytochrome c Remaining—The optical density at 550 mμ in the special spectrophotometer cell, after the reaction was complete, was decreased by the optical density reading at 550 mμ for 1 ml. of the cytochrome oxidase diluted to 3 ml. with buffer and water. The value at 550 mμ for cytochrome oxidase is within 0.008 density unit of being an isosbestic point. The corrected value (cell reading minus the cytochrome oxidase reading) was used to calculate the amount of ferrocytochrome *c*.

Ferricytochrome c Remaining—These values were obtained by difference; the values in Column 3 were subtracted from those in Column 1.

Ratio of Ferricytochrome c Remaining to Ferrocytochrome c Remaining (Column 5)—The values in Column 4 were divided by those in Column 3. The ratio in Experiment 5 must be in error, since it is not as large as would be expected, based on the progressive increase in the other values.

Ferrocytochrome c Oxidized—These values were obtained by difference; the values in Column 3 were subtracted from those in Column 2.

Fraction of Cytochrome Oxidase Reduced—The optical density readings between 575 and 625 mμ of the cell contents, after mixing, were corrected as described previously (9) for the adsorption due to the ferri- and ferrocytochrome *c* present. The corrected optical density values were plotted as in Fig. 1. Since there were isosbestic points for cytochrome oxidase at 575 and 622.5 mμ, a straight line was drawn connecting these two points on each curve. The line served as a base for calculating the increase in optical density at 605 mμ which accompanied reduction. The height of the curve above the base-line when sodium dithionite was the reducing agent, 0.084 density unit, was decreased by the height of the curve above the base-line for the oxidized preparation, 0.018 density unit. The differ-

ence is 0.066 density unit. In Experiment 2 (0.348 mg. of ferrocytochrome *c* added, Fig. 1), the values were 0.059 − 0.018 = 0.041; in Experiment 3 (0.215 mg. of ferrocytochrome *c* added) they were 0.049 − 0.018 = 0.031; and in Experiment 4 (0.113 mg. of ferrocytochrome *c* added) they were 0.041 − 0.018 = 0.023. In the other experiments (not illustrated in Fig. 1) the values were as follows: Experiment 1, 0.059 − 0.018 = 0.041; Experiment 5, 0.034 − 0.018 = 0.016; Experiment 6, 0.022 − 0.018 = 0.004. When the experimental values were divided by the difference between the value for the fully reduced sample and the fully oxidized sample, 0.066, the degree of reduction was obtained: in Experiment 1, 0.041/0.066 = 0.62. The fact that the fraction reduced is the same in Experiments 1 and 2, even though the ferrocytochrome *c* added is much greater in Experiment

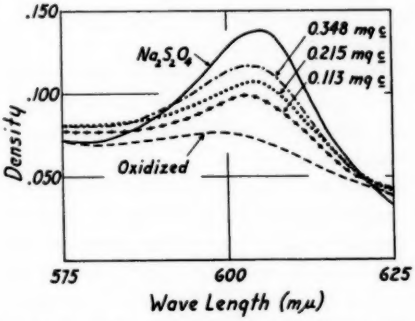

FIG. 1. Reduction of cytochrome oxidase with several concentrations of ferro cytochrome *c*.

1, suggests that this method of making the corrections is probably not valid at high concentrations of cytochrome *c*.

Fraction of Oxidized Cytochrome Oxidase Remaining—These values are the fraction of oxidized cytochrome oxidase not reduced.

Ratio of Fraction of Cytochrome Oxidase Reduced to Fraction of Cytochrome Oxidase Remaining (Column 9)—The values in Column 7 were divided by those in Column 8.

Ratio of Fraction of Cytochrome Oxidase Reduced to Ferrocytochrome c Oxidized (Column 10)—The values in Column 7 were divided by those in Column 6. The relative constancy of these ratios is evidence for the stoichiometry of the reaction.

K—The equilibrium constants at 25° were calculated according to the relationship K = (ferricytochrome *c* remaining) (fraction of cytochrome oxidase reduced)/(ferrocytochrome *c* remaining)(fraction of oxidized cytochrome oxidase remaining). The average value is 3.81.

E'_0—The oxidation-reduction potentials at 25° were calculated according to the formula $E'_0 = E'_0$ (for cytochrome *c*) + 0.05915 log (ferricytochrome *c*)/(ferrocytochrome *c*) + 0.05915 log (fraction of cytochrome oxidase reduced)/(fraction of oxidized cytochrome oxidase remaining), where E'_0 at 30° for cytochrome *c* = 0.254 volt as obtained by Rodkey and Ball (13). The average value for cytochrome oxidase is 0.285 volt. This is to be compared with the value of 0.29 volt obtained earlier by Ball (14).

$-\Delta F$—The free energy values were calculated according to the formula, $-\Delta F = RT \ln K$, where $R = 1.9864$ calories per mole per °C., $T = 298°$, and K is obtained from Column 11. The average value is 729 calories per electron. This value is to be compared with the value of 460 calories per electron at 20° calculated by Ball (15) from his oxidation-reduction potential data. The difference is largely due to the lower potential for cytochrome *c* used in the calculations here.

ΔD *at 605 mμ*—These values are the ones that were calculated above when the fraction of cytochrome oxidase reduced was discussed. They were obtained from the three corrected adsorption curves presented in Fig. 1 and from other similar curves.

$\Delta\epsilon^{l\ cm.}_{M\ per\ l.}$ *at 605 mμ*—The differences in the molar extinction coefficients for reduced and oxidized cytochrome oxidase at 605 mμ were calculated according to the formula, $\Delta\epsilon^{1\ cm.}_{M\ per\ l.} = \Delta D/cl$, based on the assumption that 1 mole of cytochrome oxidase iron is reduced per mole of ferrocytochrome *c* oxidized. ΔD is obtained from Column 13, *c* = mg. of ferrocytochrome *c* oxidized × $10^3/3$ × (1.227×10^7), and l = 1 cm. The $\Delta\epsilon$ values average 0.076×10^5 sq. cm. per gm. atom of Fe.

SUMMARY

Cytochrome oxidase (containing sodium deoxycholate) has been reduced with several concentrations of ferrocytochrome *c* (prepared with hydrogen and platinum) under conditions which result in an equilibrium. From the data it is calculated that K for the reaction equals 3.81 (range, 1.66 to 6.22) and that $-\Delta F$ equals 729 calories (range, 302 to 1090) per electron at 25°. It is also calculated that E'_0 at 25° for cytochrome oxidase, based on Rodkey and Ball's value of 0.254 volt at 30° for cytochrome *c*, equals 0.285 volt (range, 0.267 to 0.301), and that $\Delta\epsilon^{1\ cm.}_{M\ per\ l.}$ at 605 mμ equals 0.076×10^5 sq. cm. (range, 0.064 to 0.093 × 10^5) per gm. atom of Fe.

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PHYSICAL, CHEMICAL, AND IMMUNOLOGICAL STUDIES ON GLOBULINS FROM MULTIPLE MYELOMA*

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Patients with multiple myeloma frequently have a high content of serum protein because of the presence of large amounts of a globulin component. Many studies have been made of these globulins in efforts to ascertain whether the proteins represent an increase in a normal constituent or the *de novo* synthesis of an abnormal globulin (1–3).

For some years, we have been concerned with studies of the chemical nature of γ-globulins and antibodies (3–6). As a part of these investigations a chemical study of certain myeloma proteins was undertaken in the hope that this would lead to a better understanding of the nature of these proteins as well as of serum globulins in general.

Individual serum globulins from different myeloma patients may differ markedly from one another. The electrophoretic pattern of the serum is usually normal except for a superimposed protein boundary which generally possesses the mobility of a γ- or β-globulin (1), although there have been a few reports of excessive α-globulins (7, 8). In the present study, four patients were selected having myeloma globulins of very different electrophoretic mobilities. In each case, the globulin was isolated by the method of electrophoresis-convection or by a combination of this procedure with precipitation methods. The isolated proteins were then characterized by a study of electrophoretic mobility, isoelectric point, sedimentation behavior, amino acid composition, free amino groups, and immunochemical relationship as observed by their quantitative precipitation with antiserum to human γ-globulin.

Isolation and Electrophoretic Behavior

The electrophoretic patterns of the four samples of serum which are identified as *A*, *B*, *C*, and *D* in the order of increasing mobility of the abnormal globulin constituent are presented in Fig. 1. The mobility of the isolated globulins *A* to *D* was essentially the same as in the serum.

* This investigation was aided by grants from the National Institutes of Health, United States Public Health Service.

† Predoctoral Fellow of the Life Insurance Medical Research Fund.

Each of the four patients from whom serum was obtained showed the characteristic plasma cell infiltration of the bone marrow. Two of the patients have had no bone pain and have manifested no radiographic evidence of bone lesions up to the present time. However, alterations in the bone marrow in conjunction with the abnormalities in plasma protein suffice to establish a diagnosis of multiple myeloma.[1]

For the isolation of the globulins, an electrophoresis-convection apparatus (Beckman Instruments, Inc.) of the type developed by Cann *et al.* (9) was used. Samples of sera *A*, *B*, and *C* were brought to 0.5 saturation

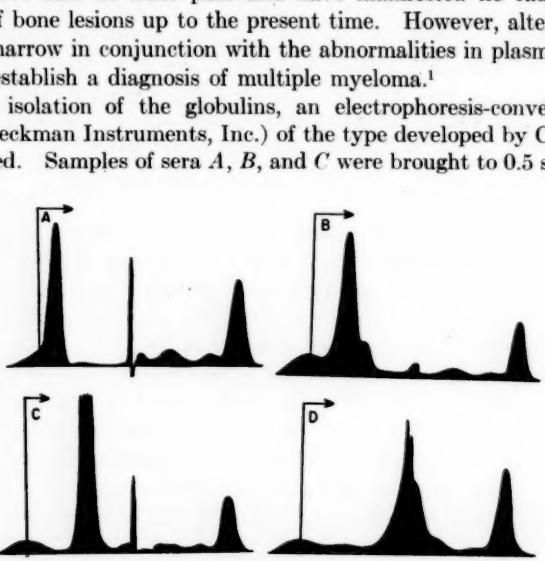

FIG. 1. Descending patterns after 250 minutes of the sera of four patients with multiple myeloma as determined by electrophoretic analysis at 1° in 0.1 ionic strength Veronal buffer at pH 8.4 to 8.5. The protein concentration was 1.6 to 1.7 per cent. The letters (*A* to *D*) are given in the order of increasing mobility of the globulin component present in large amounts. The same letters are used to designate the isolated myeloma globulins.

with $(NH_4)_2SO_4$. The dialyzed precipitates were run in the convection apparatus at 3.0 to 3.6 per cent protein in phosphate buffer at 0.1 ionic strength and pH 7.4 for 48 hours at 1.6 to 1.8 volts per cm. The material in the top compartment was nearly or completely homogeneous after one or two runs (Fig. 2, *A*, *B*, and *C*). Additional globulin could also be recovered by rerunning the preparations recovered from the middle and bottom sections of the cell under the same conditions. From 0.5 to 1.1 gm. of each of these three globulins was obtained by this method.

For the isolation of protein *D*, a β-globulin, a somewhat different procedure had to be employed. The serum was brought to 0.5 saturation

[1] We are greatly indebted to Dr. A. P. Macfarlane and Mr. Donald Smith for their cooperation in obtaining blood samples from two of the myeloma patients.

with $(NH_4)_2SO_4$ and the precipitate dialyzed against the phosphate buffer. The preparation was then run by electrophoresis-convection under the conditions given above. At the end of the run, there was little protein in the top chamber. The bottom fraction revealed, by analysis at pH 8.5 (Veronal buffer) in the Tiselius apparatus, 61 per cent of the β component, 6 per cent γ-globulins, 11 per cent α-globulins, and 22 per cent albumin. This material was precipitated at 0.3 saturated $(NH_4)_2SO_4$; after dialysis 91 per cent β-globulin and 9 per cent albumin were found. In 0.15 M NaCl a fraction was precipitated at 25 per cent ethanol at $-5°$; after lyophilization, the product proved to be homogeneous (Fig. 2, *D*).

For physical and immunological studies, samples of the four proteins were dried from the frozen state. The dried proteins were then dialyzed

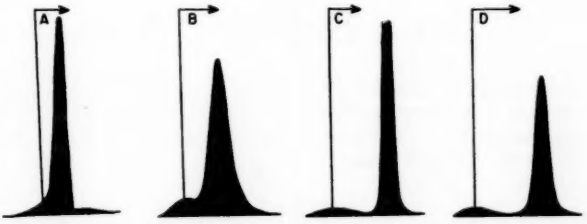

FIG. 2. Descending patterns of the four isolated myeloma globulins after electrophoretic migration in 0.1 ionic strength Veronal buffer at pH 8.4 to 8.5. The conditions were as follows: *A*, 250 minutes at 0.9 per cent; *B*, 250 minutes at 1.5 per cent; *C*, 225 minutes at 1.1 per cent; and *D*, 150 minutes at 0.9 per cent.

in the cold against appropriate buffer solutions. For studies of free amino groups and amino acid composition, the proteins were precipitated with 50 per cent ethanol, washed with 95 per cent ethanol until the washings were free of inorganic ions, and then dried by washing three times each with absolute ethanol and with ether. The moisture content of the samples equilibrated in air was determined in the usual manner.

Representative electrophoretic patterns of the four globulins appear in Fig. 2. The preparations were studied over a wide range of pH. Protein *A* revealed a few per cent of normal γ-globulin. The other proteins proved to be essentially homogeneous over the pH range shown in Fig. 3. From these results, it is evident that the method of electrophoresis-convection is almost ideal for the isolation of the myeloma globulins, particularly for those of low mobility.

The electrophoretic mobility of the four proteins as a function of pH is illustrated in Fig. 3. It is apparent that the four proteins differ markedly. The isoelectric points are reported in Table I.

Sedimentation Behavior

The isolated globulins were studied in the Spinco ultracentrifuge with the controls and procedures previously described (10–12). The runs were

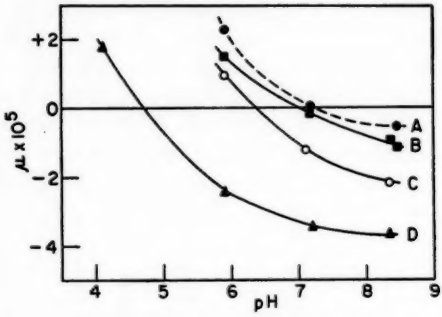

FIG. 3. Electrophoretic mobility as a function of pH for the four myeloma globulins determined at 1° in univalent buffers at 0.1 ionic strength. The pH was measured at room temperature. The buffers used were acetate at pH 4.1 and 5.9 and Veronal-NaCl at the other pH values.

TABLE I

Physical Properties of Four Myeloma Globulins

Electrophoretic mobility (u) is given in sq. cm. per volt per second. The isoelectric point was determined at 1° in univalent buffers at 0.1 ionic strength. $s'_{20,w}$ is the sedimentation constant extrapolated to zero concentration, in Svedberg units.

Protein	u × 10^5 at pH 8.4	Isoelectric point	Main component		Secondary components,* $s_{20,w}$
			$s'_{20,w}$	Estimated mol. wt.	
A	−0.5	7.2	6.58	160,000	9.4 (15%)
B	−1.0	7.05	9.66	320,000	6.6 (8%), 13.3 (11%)
C	−2.1	6.4	6.53	160,000	8.3 (11%)
D	−3.6	4.7	6.56	160,000	8.3 (19%), 11.0 (22%)

* Estimated at the following concentrations: *A*, 1.5 per cent; *B*, 0.8 per cent; *C*, 1.2 per cent; and *D*, 0.8 per cent. The amount of each component is given parenthetically.

made at 59,780 r.p.m. at room temperature (20–25°), and the globulins were equilibrated with Veronal-NaCl buffer at pH 8.5, 0.15 ionic strength. A representative pattern for each protein is illustrated in Fig. 4. These runs indicate that the preparations were not entirely monodisperse, a situation frequently encountered with γ-globulins (3). For proteins *A*, *C*, and *D* the main component had a sedimentation constant ($s_{20,w}$) of about

6.6 Svedberg units (S) (Fig. 5); for the heavier material, $s_{20,w}$ was about 9 to 10 S. For the main component of protein *B*, $s_{20,w}$ = 9.7 S at zero concentration. Such differences in $s_{20,w}$ for myeloma globulins have been reported earlier (1, 2, 13). In order to be certain that the high $s_{20,w}$ of

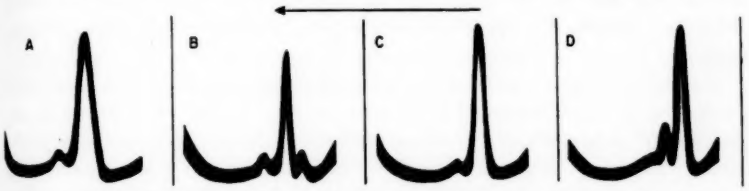

FIG. 4. Sedimentation patterns for four isolated myeloma proteins at 59,780 r.p.m. The conditions were as follows: protein *A*, 47 minutes after reaching full speed at 1.5 per cent; protein *B*, 30 minutes after reaching speed at 1.0 per cent; protein *C*, 44 minutes and 1.2 per cent; and protein *D*, 32 minutes and 0.8 per cent.

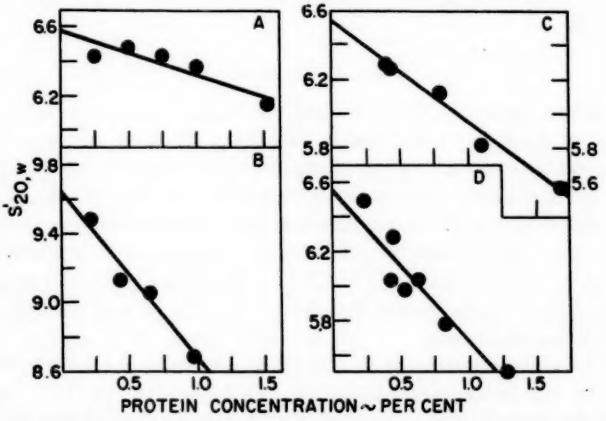

FIG. 5. Sedimentation constant ($s_{20,w}$) as a function of concentration for the main component of each of the four myeloma proteins. The lines drawn through the data were calculated by the method of least squares.

globulin *B* was not an artifact caused by the method of preparation, fresh, untreated samples of serum *B* were examined in the ultracentrifuge. The dilutions were made with Veronal-NaCl at pH 8.5 and 0.1 ionic strength to a protein concentration of about 1 per cent. The component with s = 8.5 S (at 1 per cent protein) represented 68 per cent of the total sedimenting area. Protein *B* represented 57 per cent of the total serum protein as estimated electrophoretically.

Fig. 5 contains the $s_{20,w}$ values at different protein concentrations for

the main component of each preparation. Proteins *A*, *C*, and *D* yield sedimentation constants at zero concentrations, $s'_{20,w}$, which are in excellent agreement with those found for the main component of normal human γ-globulins (14) and many myeloma globulins (13); these proteins, like normal γ-globulins, are estimated to have a molecular weight of about 160,000. Protein *B* is probably twice this size, namely 320,000 (see below).

It is noteworthy that the slope of the lines for $s_{20,w}$ as a function of concentration is much steeper for proteins *D* and *C* than for protein *A*, although the extrapolated values at zero concentration are essentially the same. This may be a reflection of the high net charge of the more acidic proteins which might be expected to show a greater primary charge effect at pH 8.5 than would protein *A*, which is isoelectric at pH 7.2. The slope and $s'_{20,w}$ for protein *A* are almost identical with those found by Putnam and Udin (13) for similar γ-globulins from myeloma patients and by Cann (14) for normal γ-globulins isolated by the method of electrophoresis-convection.

Free Amino Groups

The dinitrophenyl (DNP) method of Sanger (15) provides a useful criterion for the study of protein homogeneity (16) and, at the same time, gives an estimate of the number of peptide chains and the size of a protein. Studies by McFadden and Smith (6) and by Putnam (17, 18) of normal human γ-globulins have shown that different fractions differ in *N*-terminal residues, but usually contain *N*-terminal aspartic and glutamic acids. One myeloma protein has been reported to have the same end-groups (6), but Putnam (17, 18) has found that different myeloma proteins may possess other *N*-terminal groups.

The four globulins isolated in this study were examined with the DNP method by the procedures utilized in this laboratory (5, 19). Approximately 100 mg. or more of each DNP protein were hydrolyzed at 105° for 18 hours in 6 N HCl. DNP amino acids were identified by chromatography on paper in two or more solvent systems (2). Quantitative estimations of the DNP derivatives were made with the Beckman spectrophotometer after separating them on buffered Celite columns (19, 20). All of the usual precautions were followed, and corrections for the destruction of DNP amino acids were made with the factors given by Sanger and Porter (21). The results for proteins *A*, *B*, *C*, and *D*, for the cryoglobulin obtained from a case of multiple myeloma studied earlier (6), and for two fractions of normal human γ-globulin are reported in Table II. The normal human γ-globulins were preparations obtained from large pools of normal serum by alcohol fractionation methods (23); samples of the identi-

cal preparations have been used earlier for a number of chemical and immunological studies (3, 22, 24).

The end-group studies indicate that these myeloma proteins differ from one another and that they possess a high degree of chemical homogeneity. The failure to detect DNP-aspartic acid in proteins *A*, *B*, and *C* or DNP-glutamic acid in protein *D* indicates the virtual absence of other types of γ-globulins in these preparations. Protein *B* probably consists of four peptide chains, each with *N*-terminal glutamic acid, to satisfy the estimated

TABLE II

Free Amino Groups of Myeloma Globulins

Protein	Moles amino acid per 160,000 gm. protein			
	Aspartic acid	Glutamic acid	Lysine (by DNP)	Lysine (by chromatography)*
Myeloma *A*		2.1	99	93
" *B*		2.01	70	66
" *C*		2.86	93	93
" *D*	1.04		68	68
Cryoglobulin†	1.49	1.53	69	
γ-Globulin II-1,2‡	1.06	1.82	75	79§
γ-Globulin II-3‖	1.01	1.00	73	69§

* Calculated from data in Tables III and IV.

† The properties of this myeloma globulin have been reported earlier (6), together with amino group determinations on a less pure preparation.

‡ Average of five determinations; also obtained, 0.10 mole of DNP-serine.

§ Microbiological estimations reported earlier (22).

‖ Average of seven determinations; also obtained, 0.17 mole of DNP-serine. The average values given here differ slightly from those reported earlier (6) because of the inclusion of additional determinations.

molecular weight of 320,000. Proteins *A* and *C* differ in the number of *N*-terminal glutamic acid residues per mole, whereas protein *D* reveals only one *N*-terminal aspartic acid residue per mole of protein.

It is noteworthy that the four myeloma proteins in Table II differ not only from one another but also from most of those studied by Putnam (17, 18). This investigator described proteins with 2 *N*-terminal residues of aspartic acid per 160,000 gm., with 2 *N*-terminal leucine (or isoleucine) residues, and with equimolar amounts of terminal aspartic and glutamic acids in a sample of a β-globulin. He also found one globulin, similar to protein *C*, with 3 residues of *N*-terminal glutamic acid per mole.

The lysine content of some of the myeloma proteins is also strikingly different. Values for ε-DNP-lysine are in good accord with the chroma-

tographic estimations of the lysine content of these proteins. While our work was in progress, Grisolia and Cohen (25) reported that certain myeloma proteins showed variations in content of lysine and other amino acids as estimated by microbiological analysis.

In view of the presence of multiple peptide chains in myeloma proteins *A*, *B*, and *C*, it was of interest to study partial hydrolysates of some of the DNP proteins in order to determine *N*-terminal sequences. With the limited available material, only a few preliminary studies have been possible thus far. The partial hydrolysis was performed in 12 N HCl for 4 or 5 days at 37°. From protein *B*, two DNP peptides were separated in modest yield by chromatographing partial hydrolysates on buffered Celite columns. After complete hydrolysis, one DNP peptide gave DNP-glutamic acid and free alanine; the other yielded DNP-glutamic acid and free leucine (or isoleucine). These compounds must have been DNP-glutamylalanine and DNP-glutamylleucine (or isoleucine). This finding indicates that at least two of the peptide chains of protein *B* are different.

From a partial hydrolysate of the DNP derivative of protein *A*, only a single dipeptide could be obtained in low yield; this substance proved to be DNP-glutamylleucine (or isoleucine). This peptide may be identical with the similar one obtained from protein *B*. It is apparent, however, that further studies are necessary to determine possible chemical relationships among various myeloma proteins.

Amino Acid Composition

When this investigation was initiated, no studies had been reported of the chemical composition of myeloma globulins. Detailed analyses were undertaken and these have revealed many interesting differences among the four proteins. In the meantime, the studies of Grisolia and Cohen (25) on eight myeloma proteins have appeared, and their results also reveal differences among the proteins analyzed by them.

Analyses were made by chromatography of acid hydrolysates on Dowex 50-X8 by the methods of Moore and Stein (26, 27). Calculations, procedures, and controls as performed in this laboratory have been reported previously (28, 29). Representative elution curves which demonstrate some of the differences among the proteins are illustrated in Figs. 6 and 7.

Table III gives the average data for the amino acids determined by chromatography and for the separate determinations performed in duplicate by other methods. Tryptophan was determined by a colorimetric method on the unhydrolyzed protein (30). Cysteic acid was estimated on the hydrolysates of the oxidized proteins by the procedure of Schram, Moore, and Bigwood (31); the amino acid was isolated by chromatography on a 0.9 × 15 cm. column of Dowex 50-X8. Hexose content was determined by the orcinol method (32), with glucose as the standard; absorb-

ancy was measured at 420 mμ. Hexosamine was measured by the procedure of Palmer *et al.* (33), with glucosamine as the standard; readings were made at 530 mμ. The Coleman junior spectrophotometer was used for all the colorimetric estimations.

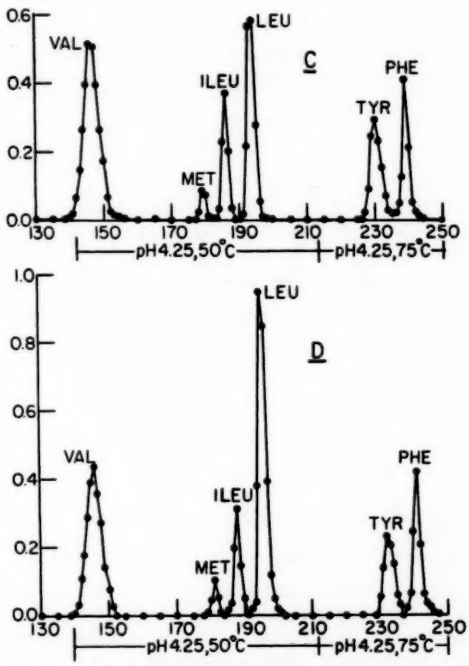

FIG. 6. Portions of the elution curves for 70 hour hydrolysates of myeloma proteins *C* (1.39 mg. of protein) and *D* (1.50 mg. of protein) determined by the ninhydrin method after separation on Dowex 50 columns (0.9 × 100 cm.). Ordinates, optical density, corrected for base-line color, but uncorrected for different color yields of the amino acids; abscissae, number of 1 ml. fractions collected. Note especially the different yields of valine (VAL), isoleucine (ILEU), leucine (LEU), and tyrosine (TYR). MET = methionine; PHE = phenylalanine.

Determinations of the amide content were performed as described previously (19). Duplicate values agreed within 4 per cent of one another. The amide determinations which were made by estimation of the ammonia separated on Dowex 50 are omitted as being less reliable than the direct determinations; however, the two sets of values agreed within 10 per cent.

In view of the differences in the isoelectric points of the myeloma proteins, the great variations in composition reported in Table III are not surprising. It is noteworthy that the variations extend to almost every

amino acid constituent and to the carbohydrate[2] components as well; however, the essentially constant level of histidine is striking. Nevertheless, all of the values are not too different from reported analyses of human γ-globulins (3), and the individual myeloma proteins resemble the less complete analyses obtained by microbiological assays in the study of Grisolia and Cohen (25).

As already noted (25), the serum myeloma proteins all contain methionine. In addition to other distinguishing features, this finding clearly

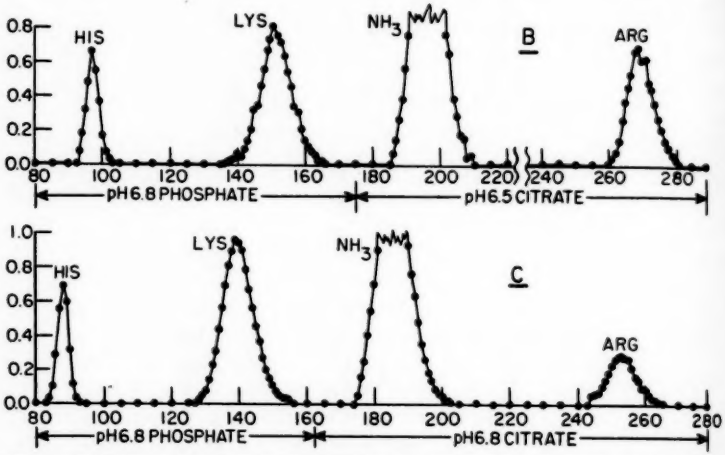

FIG. 7. Elution curves for the basic amino acids of proteins *B* (7.31 mg. of protein) and *C* (6.93 mg. of protein) for 70 hour hydrolysates. The ordinates and abscissae are the same as in Fig. 6. Note the differences in amount of arginine (ARG) and lysine (LYS) of the two proteins. HIS = histidine.

differentiates them from Bence-Jones proteins, in which this amino acid is lacking or present at negligible levels (38–40).

Noteworthy differences in the four proteins are revealed by a comparison of their ionic groups. Table IV gives, for a molecular weight of 160,000, the number of residues of each amino acid which contribute ionic groups

[2] The presence of carbohydrate, estimated as "hexose and hexosamine" in γ-globulins and antibodies of various species including man, has been established earlier (4, 34–36). Osserman and Lawlor (37) have recently reported that myeloma proteins of serum, separated by electrophoresis on paper, give a characteristic carbohydrate test, but they did not find carbohydrate in the γ-globulin of normal serum. It should be noted that myeloma proteins *A*, *B*, and *C* of the present study contain about the same amounts of carbohydrate that were found earlier in fractions of normal γ-globulin (36).

in the isoelectric region of each globulin. For protein *A*, the composition is in accord with the measured pI (isoelectric point) of 7.2. At this pH

TABLE III

Amino Acid Composition of Four Myeloma Proteins

The data are presented as gm. of residue per 100 gm. of anhydrous protein. Values for the first sixteen amino acids are based on duplicate determinations of preparations hydrolyzed for 20 and 70 hours. All threonine and serine values and the methionine content of protein *C* were estimated by extrapolation of the recoveries to zero hydrolysis time. Isoleucine and valine determinations are the averages for the 70 hour hydrolysate only.

Residue	Protein *A*	Protein *B*	Protein *C*	Protein *D*
Aspartic acid	8.48 ± 0.14*	7.65 ± 0.22*	8.19 ± 0.17*	7.28 ± 0.18*
Threonine	6.41 ± 0.07	8.49 ± 0.19	6.40 ± 0.14	7.92 ± 0.04
Serine	10.16 ± 0.04	9.10 ± 0.14	10.54 ± 0.33	7.81 ± 0.02
Glutamic acid	10.58 ± 0.17	11.30 ± 0.45	10.69 ± 0.35	10.09 ± 0.12
Proline	5.94 ± 0.05	7.14 ± 0.32	6.64 ± 0.27	6.61 ± 0.25
Glycine	3.78 ± 0.14	3.84 ± 0.03	3.61 ± 0.06	3.24 ± 0.10
Alanine	3.88 ± 0.10	4.93 ± 0.26	4.69 ± 0.17	4.36 ± 0.05
Valine	8.44 ± 0.33	6.96 ± 0.04	8.60 ± 0.09	6.18 ± 0.12
Methionine	0.97 ± 0.06	0.98 ± 0.06	1.38 ± 0.02	0.80 ± 0.04
Isoleucine	2.30 ± 0.06	2.56 ± 0.05	2.94 ± 0.01	2.27 ± 0.01
Leucine	8.24 ± 0.18	9.14 ± 0.28	5.80 ± 0.08	8.38 ± 0.08
Tyrosine	6.66 ± 0.22	4.96 ± 0.10	6.05 ± 0.06	4.38 ± 0.13
Phenylalanine	4.78 ± 0.15	4.92 ± 0.16	4.37 ± 0.10	4.62 ± 0.10
Histidine	2.12 ± 0.03	2.01 ± 0.01	2.22 ± 0.06	2.10 ± 0.06
Lysine	7.42 ± 0.12	5.33 ± 0.03	7.44 ± 0.26	5.44 ± 0.21
Arginine	4.91 ± 0.21	5.07 ± 0.22	2.72 ± 0.05	4.96 ± 0.08
Ammonia†	1.26 ± 0.02	1.31 ± 0.02	1.20 ± 0.02	1.51 ± 0.04
Tryptophan	1.48 ± 0.19	1.51 ± 0.09	2.84 ± 0.02	2.45 ± 0.03
Half cystine	1.83 ± 0.03	2.48 ± 0.07	1.77 ± 0.04	2.27 ± 0.06
Hexose	1.59 ± 0.03	0.83 ± 0.05	1.11 ± 0.02	3.99 ± 0.05
Hexosamine	0.30 ± 0.01	0.81 ± 0.01	1.32 ± 0.02	3.10 ± 0.10
Total weight recovery	100.27	100.01	99.32	98.25
Total N recovery in gm.	16.48	16.52	16.01	15.90

* Average deviations.

† Omitted from the total weight recovery.

most of the imidazole groups would be uncharged and the protein would be slightly basic. This is also true for protein *B* whose pI = 7.05. For protein *C*, pI = 6.4, and at this pH approximately half of the imidazole groups might be expected to be in the cationic form. From this one would

predict a slightly basic protein. If all of the imidazole groups were uncharged at pH 6.4, the discrepancy would vanish and there would be a slight excess of anionic groups.

For protein *D*, the discrepancy between the measured pI of 4.7 and the amino acid composition is great indeed. There is a calculated excess of 68 basic groups, and, regardless of the pK values assigned to the twenty-four imidazole groups, the protein should possess a basic rather than a markedly acidic pI value. The analyses cannot be in error by amounts sufficient to explain the discrepancy. It may be noted that all of the ϵ-amino groups of lysine must be free, since the estimations by the DNP method are in good agreement with the chromatographic estimations of

TABLE IV

Ionic Groups of Myeloma Proteins

The number of groups has been computed per 160,000 gm. of each protein.

Amino acid	Protein *A*	Protein *B*	Protein *C*	Protein *D*
Aspartic acid	118	106	114	101
Glutamic "	131	140	132	125
Amide groups	126	131	120	151
Free anionic groups	123	115	126	75
Arginine	50	52	28	51
Lysine	93	66	93	68
Histidine	25	23	26	24
Free cationic groups	168	141	157	143
Excess cationic groups	45	26	21	68

this amino acid on the hydrolyzed proteins (Table II). It must be concluded that for protein *D* (and perhaps to a lesser extent for protein *C*) other factors are involved.

The composition of protein *D* differs markedly from the other myeloma proteins in the large amount of carbohydrate estimated as "hexose" by the orcinol method (as well as in hexosamine). Although other acidic substances may be present in small amount, there is the possibility that the "hexose" present in this glycoprotein may be acidic in nature. Hexuronic acids react with the orcinol reagent to yield colors equivalent to those given by hexoses. The data indicate that approximately 40 "hexose" residues are present, and, if all or nearly all of these residues are acidic, the discrepancy between the amino acid composition and the pI would be greatly diminished. Further studies are now in progress on protein *D* and similar proteins in order to determine the nature of the acidic components.

Precipitin Studies

Two adult, female albino rabbits (2.8 and 3.2 kilos) were injected intravenously three times weekly for 5 weeks with 2.0 mg. of Fraction II-1,2 of human γ-globulin. 3 days after the final injection, the animals were bled and the sera pooled. The 40 ml. of serum were absorbed by addition of 0.5 ml. of serum from a patient with agammaglobulinemia. After 4 days in the cold the precipitate was removed. The antiserum was then fractionated by precipitating first with 0.4 and then with 0.35 saturated ammonium sulfate. The globulin precipitate containing antibody was dialyzed until free of sulfate and dissolved in 0.85 per cent NaCl containing merthiolate as a preservative.

For quantitative precipitin studies, 0.5 ml. of antigen in 0.85 per cent NaCl was added to 0.5 ml. of antibody solution. The tubes were frequently mixed over a period of 72 hours and the precipitate was separated and washed twice with 3.0 ml. of saline by centrifuging at 4°. The quantity of precipitate was estimated with the Folin-Ciocalteu reagent or by a micro-Kjeldahl method, or by both procedures. The factor 6.25 was used for converting protein N of the antigen to protein. The supernatant solutions were tested by addition of Fraction II-1,2 to an aliquot in one tube, and of the myeloma globulin being used as antigen in a second tube, and of the antiserum to Fraction II-1,2 in a third.

Plots were made of the ratio of antibody to antigen *versus* antigen added in the region of excess antibody, and the method of least squares was applied to the linear plots obtained. The amount of antibody precipitated at the zone of equivalence was calculated from the equation of Heidelberger and Kendall (41) and compared with the observed value.

The results in Table V show that none of the myeloma globulins is identical with Fraction II-1,2 antigen when examined with the antiserum. It is also evident that protein *D*, which is a β-globulin, is the least related of all. This protein also deviated greatly from the other three in isoelectric point and chemical composition. It should be noted that the preparations of proteins *A*, *B*, and *C* were identical with those used for the physical and chemical studies, and, as already noted, protein *A* contained a few per cent of normal γ-globulin. Unfortunately, the supply of protein *D* used for all of the studies described above had been exhausted. The new preparation, made by essentially the same method, contained 91 per cent of the β-globulin and 9 per cent of γ-globulin, with the usual electrophoretic mobility.[3]

[3] The failure to obtain a homogeneous preparation of protein *D* by repeating the first procedure was undoubtedly due to the fact that a much smaller quantity of serum had to be used. The fractionation by electrophoresis-convection is less successful at low protein concentrations (9). Only 0.3 gm. of protein *D* was obtained

From one to three quantitative estimations of protein precipitate were made in the zone of antigen excess (3 to 10 times the amount of antigen present at the zone of equivalence) with this antiserum and with Fraction II-1,2 and the four myeloma proteins as antigens. In no instance was there a striking decrease from the observed maximal precipitate at equivalence in the zone of antigen excess (*cf.* Cohn *et al.* (42)).

The supernatant fluid of antiserum treated with varying amounts of Fraction II-1,2 or the myeloma proteins gave precipitates either with added antigen or added antiserum but not with both. With the chemically homogeneous proteins *B* and *C*, analyses of the supernatant fluids by

TABLE V

Precipitin Reactions of Myeloma Globulins with Antiserum to γ-Globulin

The results are given per ml. of arbitrarily diluted antibody solution. The amount of antibody precipitated (*Ab*) was calculated from the equation (41) $(Ab/An) = 2R - (R^2An/A)$, where *An* is the antigen added, *R* is the ratio of antibody to antigen at equivalence, and *A* is the total antibody in the system.

Antigen	Antibody pptd.	
	Calculated	Found
	mg.	*mg.*
Fraction II-1,2	1.94	1.91
Myeloma protein *A*	1.26	1.28
" " *B*	0.86	*
" " *C*	1.32	1.43
" " *D*	0.58	*

* These were not carried to the zone of equivalence.

addition of excess amounts of these antigens still yielded strong precipitates when Fraction II-1,2 was added. This is further evidence that these myeloma proteins did not remove all of the antibody from the antiserum and is in good agreement with the quantitative studies.

Analysis revealed that after absorption with an excess of myeloma protein *A*, which contained some γ-globulin, the antiserum contained no residual antibody which would yield a precipitate with Fraction II-1,2. The 9 per cent γ-globulin in the preparation of protein *D* did not interfere significantly with the amount of precipitate formed in the zone of antibody excess. However, when the antiserum was absorbed with an excess of this preparation *D* (6 times the amount of antigen calculated to be present at the zone of equivalence), there was an increase in the amount of pre-

from 20 ml. of serum in the second fractionation; this was insufficient to attempt removal of the 9 per cent γ-globulin by further fractionation.

cipitate; this could be ascribed to the contaminating γ-globulin. In this instance the supernatant solution gave no precipitate when Fraction II-1,2 was added. From the quantitative studies, it appears highly unlikely that a homogeneous preparation of protein *D* would remove all of the antibody to Fraction II-1,2 from this antiserum.

A sample of antiserum to Fraction II-1,2 was repetitively absorbed first with protein *B*, then with protein *C*, and then with Fraction II-1,2. In each instance, 0.5 mg. of antigen protein was added per ml. of antiserum. This is sufficient antigen to place the reaction in the region of antigen excess. This was confirmed in each instance by the finding of no further reaction when more antigen was added to an aliquot of the supernatant fluid. The precipitin reactions were performed as described above. The results are presented in Table VI.

TABLE VI

Repetitive Absorption of Antiserum to γ-Globulin

Antigen	Ppt. for successive absorptions*	Ppt. with unabsorbed antiserum at equivalence
	mg.	*mg.*
Protein *B*	0.66	1.00
" *C*	0.34	1.67
Fraction II-1,2	1.15	2.34

* Average of duplicate determinations.

It will be noted that the amounts of precipitate removed from the antiserum were less than at the zone of equivalence; this is to be expected. The amount of precipitate obtained by addition of protein *C* after previous absorption with protein *B* was much less than anticipated. This finding suggests that proteins *B* and *C* have some common antigenic properties, although protein *C* must have some antigenic characteristics distinct from those of protein *B*.

Kunkel *et al.* (43) previously noted the occurrence of cross-reactions between myeloma globulins and human γ-globulin. This work has been amplified in a recent report by Slater, Ward, and Kunkel (44) in which twenty-one myeloma proteins (β or γ) were examined for their immunochemical relationships to one another and to human γ-globulin. With the exception of one β-myeloma globulin, each of the other myeloma proteins (β and γ) gave precipitates when added to rabbit antiserum to human γ-globulin, although wide variations occurred in the extent of cross-reactivity. In general, β-myeloma proteins removed much less antibody from antiserum to human γ-globulin than did the γ-myeloma globulins. No

two myeloma proteins showed identical behavior with the quantitative studies. Similarly, antisera to certain β- and γ-myeloma proteins showed varying reactivity with other myeloma proteins and with normal γ-globulin. The wide variation in myeloma proteins and the absence of complete immunochemical identity of any one of the twenty-one proteins with another are remarkable. Wuhrmann and coworkers (45) also have noted that myeloma proteins of high electrophoretic mobility do not cross-react with antisera to normal γ-globulin. Deutsch (46) has observed a quantitatively different reaction for chicken antiserum to human γ-globulin when the antigens are normal γ-globulin or three γ-myeloma globulins.

The present study, limited to four myeloma proteins of varying mobilities, also demonstrates that myeloma proteins vary quantitatively in their reactivity with an antiserum to human γ-globulin (Fraction II-1,2). The antiserum, in this instance, was absorbed with the serum of a patient with agammaglobulinemia. Previous experience of one of us (47) has indicated that such absorbed antisera react more specifically with the γ-globulin component of human serum than does unabsorbed antiserum, such as that used by Deutsch (46). Martin and coworkers (48) have demonstrated recently that antiserum prepared to human γ-globulin reacts not only with the γ-globulin of human serum, but also with a β-globulin which is present in the serum of adults but not of infants. Slater *et al.* (44) refer to unpublished work which is in accord with these findings. This may explain in part why a myeloma β-globulin would cross-react with γ-globulin. However, this alone does not suffice. The antiserum used by us was absorbed with adult serum of a patient with agammaglobulinemia. Although the effect of this remains to be proved, it is likely that antibody to the β component of normal serum, which cross-reacts with the antiserum to γ-globulin, was removed by absorption with the agammaglobulinemia serum.

DISCUSSION

The present studies on myeloma globulins are in agreement with many earlier observations that serum proteins from different patients with the same clinical disorder may show marked differences in size, isoelectric point, mobility, and immunochemical relationship to normal γ-globulin (1–3). To these properties may now be added our observations and those of Grisolia and Cohen (25) that the different globulins also exhibit striking variations in chemical composition. The present results also supplement the preliminary reports of McFadden and Smith (6) and of Putnam (17, 18) that the *N*-terminal groups of different fractions of γ-globulins and of myeloma globulins show distinct differences.

All of the present results favor the hypothesis, proposed by many investigators, that the myeloma serum globulins are probably individual, normal γ-globulins which arise in abnormal amount because of the excessive proliferation of plasma cells. There seems to be little or no evidence to support the view that the myeloma globulins are "abnormal."[4] Any one myeloma protein appears to be closely related to some fraction of normal γ-globulin, and it has been demonstrated in many laboratories that normal human γ-globulin is a mixture of closely related proteins which can be fractionated by various procedures (3, 49). Proteins with the chemical and immunological properties of γ-globulin have been described which have an electrophoretic mobility in the range of β-globulins. The observations that some β-myeloma globulins give no immunological cross-reaction with antisera to normal γ-globulin (43, 45), or only a minimal reaction as observed in the present study, could perhaps be explained by our finding that the more acidic β-globulin is much richer in carbohydrate than are the more common γ-myeloma globulins. The high carbohydrate content may be expected to alter markedly the antigenicity of the protein. If the amino acid composition of the β-globulin (protein *D*) is compared with the other globulins analyzed by us (Table III), it is clear that most of the differences in composition are due to the approximately 7 per cent carbohydrate which is present. It is also evident, as already mentioned, that the more acidic isoelectric point of protein *D* may be due to an acidic carbohydrate component, since it certainly cannot be ascribed to any alteration in the amino acid composition.

Studies of the amino acid composition of γ-globulins (3) and antibodies (4) from different species show that all of these proteins are closely related. The values for different fractions of "normal" γ-globulins which have been analyzed are very similar to those obtained on the myeloma proteins. Families of closely related proteins in different species which show differences in amino acid composition are well known; *e.g.*, serum albumins (50), insulins (51), and seed globulins (52), to cite only a few cases. What is unique about the γ-globulins is that such a variety of proteins is present normally in the same individual and that in multiple myeloma abnormal production of usually only a single type of protein occurs. Perhaps the most closely analogous situation is the case of the several known types of abnormal hemoglobins present in different individuals. However, the analytical data for normal and sickle-cell hemoglobins do not show striking differences (53), although such differences are known for the hemoglobins of different species (54–56).

[4] The discussion here pertains to myeloma serum proteins with a molecular weight of 160,000 or greater. The small proteins of the Bence-Jones type, which have been found in serum on some occasions, are not under consideration.

SUMMARY

1. Globulins of widely differing electrophoretic mobility from four patients with multiple myeloma have been isolated by the method of electrophoresis-convection or by a combination of this procedure with precipitation methods.

2. The isolated proteins differ greatly in electrophoretic mobility and isoelectric point. The main components of three of the proteins have a molecular weight of approximately 160,000, the other of 320,000.

3. The free amino groups of these proteins and of fractions of normal human γ-globulin were determined by the dinitrophenyl method. The different proteins show distinct differences from one another and from most of the myeloma proteins studied earlier. From one myeloma protein, two different DNP-glutamyl dipeptides were isolated from partial hydrolysates; this shows that at least two of the peptide chains must be different.

4. The amino acid composition of the four proteins was determined mainly by chromatography of hydrolysates on Dowex 50. The proteins differ from one another in composition but resemble normal γ-globulins. A β-myeloma globulin contains much more carbohydrate than the γ-myeloma proteins do. The acidic isoelectric point of the β-globulin at pH 4.7 is not in agreement with the amino acid composition, and it is suggested that acidic carbohydrate components may be present.

5. Precipitin studies with rabbit antiserum to a fraction of normal γ-globulin show that all the myeloma proteins react in varying degree with the antiserum, but to a lesser extent than does normal γ-globulin. The proteins are immunologically related, but are not identical with one another.

6. The relationship of the serum myeloma proteins to normal γ-globulin is discussed in relation to the view that individual myeloma globulins are probably normal components of serum which are present in excessive amounts.

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FREE AMINO GROUPS OF EQUINE γ-GLOBULINS AND A SPECIFIC ANTIBODY*

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Studies of the free amino groups of normal γ-globulin and of various antibodies of the rabbit have demonstrated that these proteins consist of a single peptide chain with *N*-terminal alanine (1, 2). Indeed, the *N*-terminal pentapeptide sequence (1, 2) and, within experimental error, the amino acid composition (3) of various rabbit antibodies are the same. In contrast to these findings, preliminary reports have indicated that the γ-globulins of horse, cow (4), and man (4, 5) are comprised of mixtures of proteins, inasmuch as certain fractions do not yield stoichiometric amounts of *N*-terminal residues and different fractions yield different amounts of end-groups.

Most of the present theories concerning the origin of antibodies are in accord with the view that γ-globulins and antibodies arise from a common polypeptide precursor, the specific configuration of the antibody arising somehow under the influence of antigen to give a complementary structure (6). In view of the existence of several chemically different types of γ-globulins in some species, as judged by the presence of different amino end-groups, it was of interest to ascertain whether a specifically precipitated antibody would also show a mixture, or whether a single *N*-terminal group would be present, as in various rabbit antibodies.

In this paper we report studies of the free amino groups of equine γ-globulins containing some tetanal antitoxic activity and of two preparations of type III pneumococcal antibody precipitated by the specific polysaccharide. It has been found that specifically precipitated, equine antibody contains a mixture of terminal amino groups, similar to those in the γ- and T-globulins. The only significant difference is that the antibody preparations contain a somewhat higher proportion of *N*-terminal alanine.

Inasmuch as antibodies of different specificity and the normal γ-globulin of the rabbit possess the same *N*-terminal sequence, it is interesting to consider the heterogeneous nature of the equine antibody in the light of theories (6) which assume that antibody specificity is determined by sur-

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face configuration rather than by variations in amino acid composition or sequence. The following explanation might apply to man and the cow, as well as the horse. In these species, γ-globulins are mixtures which may be separated into fractions varying in chemical and physical properties. If each type of γ-globulin precursor can become an antibody, then at the appropriate hypothetical time when the antigen influences the surface configuration, each of the different precursors may be modified into antibody. Precursors of different composition might be susceptible in varying degree to conversion into antibody by a particular antigen, and hence the antibody need not be homogeneous and yet variation with different antigens becomes possible. The fact that certain antibodies can be hydrolyzed by proteolytic enzymes to produce smaller molecules which retain antibody activity indicates that the entire molecule is not essential for activity (7) and is in accord with the view that variations in antibody composition are possible. The many observations that specific antibodies in the horse may appear in electrophoretically distinct fractions and that this distribution varies with time of immunization, nature of antigen, and other factors (7, 8) are also consonant with these views and the finding that specifically precipitated antibody is a mixture of proteins.

EXPERIMENTAL

Materials and Methods

The preparation (9) and properties (10–13) of the equine γ- and T-globulins have previously been described. These proteins were isolated from animals hyperimmunized against tetanus toxin; however, the amount of antitoxin in the preparations probably represents only a small proportion of the total protein in each fraction. Electrophoretic studies (9) showed that both preparations are essentially free of other plasma proteins.

Two preparations of type III pneumococcal antibody were studied. The antibody was precipitated with the specific polysaccharide, washed three times with saline, and lyophilized. End-group studies were performed on the antibody-polysaccharide complex. Type III polysaccharide gives no amino end-groups by the techniques used (2). We are greatly indebted to Dr. Michael Heidelberger for his kindness in supplying the dried antigen-antibody preparations.

The proteins or protein-polysaccharide complexes were treated with 1,2,4-fluorodinitrobenzene by the method of Sanger (14, 15). The procedures for isolation, identification, and estimation of the dinitrophenyl (DNP) amino acids have been described in earlier publications from this laboratory (2, 16). The recovery values for the DNP amino acids determined by Sanger and Porter (17) were used in the calculations.

Results

Table I presents the estimates of the free amino groups which were found by the DNP method on the equine proteins. The values for lysine represent ε-DNP-lysine. The results are computed on the basis of a molecular weight of 160,000; the main component of the γ- and T-globulins has been found to be of this size (13).

Many studies of homogeneous proteins by the DNP method have shown that free α-amino groups are present, as might be expected, in stoichiometric amounts. The presence of α-amino groups in less than molar amounts has generally been taken as evidence of inhomogeneity of the material except for cases in which breakdown of the protein occurs under treatment with 1,2,4-fluorodinitrobenzene (18). No evidence could be

TABLE I

Free Amino Groups of Equine Globulins

The results for individual residues are in moles per 160,000 gm. of protein.

Protein	Aspartic acid	Glutamic acid	Serine	Threonine	Alanine	Valine	Leucine*	Total	Lysine
γ-Globulin	0.15	0.09	0.09	0.03	0.06	0.15	0.17	0.74	76
T-Globulin	0.19	0.07	0.16	0.09	0.14	0.16	0.14	0.81	74
Pneumococcal antibody *A*	0.23	0.10	0.07		0.44	0.18	0.09	1.11	73
Pneumococcal antibody *B*	0.23	0.18	0.14		0.38	0.18	0.08	1.19	74

* Leucine or isoleucine.

obtained of the breakdown of the proteins during the procedures. It must be concluded, therefore, that the preparations listed in Table I are inhomogeneous. This is not unexpected, since γ-globulins can be fractionated by various methods (7, 8). The results for the pneumococcal antibody preparations indicate that the specific antibody is also a mixture of different proteins. In general, the same end-groups are found in the antibody as in the γ- and T-globulins. The only noteworthy difference is the greatly increased amount of *N*-terminal alanine.

SUMMARY

The free amino groups of γ- and T-globulins and of specifically precipitated type III pneumococcus antibody of equine serum have been studied by the dinitrophenyl method. All of the proteins reveal a mixture of *N*-terminal residues in less than stoichiometric amount. The antibody gave a higher yield of *N*-terminal alanine than the other proteins.

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STUDIES ON URICASE

I. PREPARATION, PURIFICATION, AND PROPERTIES OF A CUPROPROTEIN

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Uricase was first isolated in a purified form by Batelli and Stern in 1909 (1). Since then numerous investigators, among them Keilin and Har tree (2), Davidson (3, 4), Holmberg (5), and more recently Altman *et al.* (6) and Leone (7), have published improved purification procedures. In spite of the extended and intensive investigation of the problem of purification and in spite of the fact that the best procedures available lead to a purification of the enzyme from hog liver acetone powder of some 1200-fold, these enzyme preparations are not pure by any criteria that might be employed. Further, they cannot be maintained in solution even at a very high pH, and the over-all recoveries of purified enzyme are so low that they make the isolation of sufficient amounts of enzyme for analytical and mechanism studies a prohibitive task.

The inadequacies in purification techniques are reflected in uncertainties concerning the mode of action of the enzyme. It has long been believed that uricase is a true oxidase and does not function in conjunction with any of the known oxidation-reduction coenzymes (2, 4, 5). Thus a metal-catalyzed oxidation appeared implicated. Indeed, both iron and zinc have been suggested as possible electron carriers. Davidson demonstrated the presence of iron in his preparations of $Q_{O_2} = 600$ (3), but Holmberg, using an improved purification procedure leading to an enzyme preparation about 10 times more active ($Q_{O_2} = 6000$), found only one-tenth as much iron (0.02 per cent). He showed the presence of zinc in one of his preparations, an observation later confirmed by Davidson. The zinc content increased during purification but was not proportional to enzyme activity. The purest preparation had a zinc content of 0.09 per cent. Praetorius (8) also confirmed the presence of this metal in preparation at this stage of purity, but was able to remove the metal quantitatively with BAL[1] without any decline in activity.

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[1] The following abbreviations will be used in this paper: BAL, British antilewisite, 2,3-dimercaptopropanol; EDTA, Versene, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; E, log I_0/I; CoA, coenzyme A.

Similar uncertainty surrounds the mode of action of uricase on the substrate. The apparently simple oxidative and decarboxylative conversion of uric acid to allantoin was shown to be a complex series of reactions leading to a variety of products by way of hypothetical unstable intermediates (Felix *et al.* (9), Schuler (10), and Klemperer (11)). Praetorius (8) was the first to bring to bear on this problem the powerful tools of ultraviolet spectrophotometry, while Bentley and Neuberger (12) used C^{14}- and O^{18}-labeled uric acids to bring the problem into clearer focus. The recent investigations of Canellakis and Cohen (13) have aided greatly in elucidating the oxidative pathways followed by uric acid under the influence of uricase by the use of 2-C^{14}- and 8-C^{14}-labeled uric acids.

It has been the aim of this investigation to make a systematic study of some of the outstanding problems in connection with uricase action. This, the first paper in the series, will deal with a satisfactory method of preparation of apparently pure enzyme in good yield from hog liver mitochondria, and with the demonstration of enzyme-bound copper as a component essential to uricase action.[2]

Materials—The uric acid used for routine assays was a commercial product (Amend Drug and Chemical Company, c.p.). For experiments in which metal effects were important, a sample of uric acid freed of heavy metals was obtained as follows: Tris urate was prepared by adding solid Tris base to a suspension of uric acid in distilled water until all the uric acid had gone into solution (final concentration of uric acid about 0.01 M, final pH about 8.5). The solution was freed of a small amount of impurities by filtration and passed through a column containing Dowex 50 cation resin in the Tris form (obtained by treating Dowex 50 = H^+ form with an excess of Tris base). The solution was then acidified with dilute HCl and the precipitated uric acid collected on a Büchner funnel and washed with cold alcohol and ether. The acid was redissolved in a 0.01 M solution of Tris Versenate (obtained by the neutralization of EDTA by Tris base), allowed to stand at room temperature for 1 hour, and at 0° overnight, and was then reprecipitated by means of hydrochloric acid. The precipitate containing uric acid and some EDTA was then collected as before and recrystallized from boiling water.

The various other reagents and inhibitors were commercial samples of the highest purity available. The iron-binding globulin of human plasma was a gift of Dr. Surgenor.

[2] The second paper will cover some experiments bearing on the mechanism of the conversion of uric acid to an unstable intermediate by the enzyme, while subsequent phases of the investigation will be concerned with the possible intermediates and products under a wide variety of experimental conditions, with the nature of the active Cu-binding site on the enzyme, etc.

Methods

Assay—The assay used is a modification of Kalckar's spectrophotometric method (14) studied extensively by Praetorius. A suitable amount of a freshly prepared solution of potassium urate (approximately 2.0 mg. per ml., neutralized to pH 8.0 with KOH) is added to a micro cuvette (1.0 cm. light path) containing 0.02 M borate buffer, pH 8.0, to give an E_{293} of about 0.500 in a final volume of 0.98 ml. when read against a blank consisting of the same volume of borate buffer alone. Enzyme is then added in equal volumes (0.02 ml.) to the experimental and blank cuvettes, and the decrease in E_{293} (ΔE_{293}) determined at suitable intervals (15 or 30 seconds). If the ΔE_{293} per minute is less than 0.100, the rate is zero order for about 3 minutes or more after the first 30 seconds, and, since it is also strictly proportional to enzyme concentration in dilute solutions, this zero order rate provides a satisfactory assay for the enzyme from the first crude extracts to the stage of final purity.

Units and Specific Activity—1 enzyme unit is defined as that amount which will give rise to a ΔE_{293} equal to 1.00 per minute under the conditions just described. Since the molar extinction coefficient of uric acid equals 12.3×10^3 (15) under the same conditions of pH, buffer composition, and ionic strength, 1 unit corresponds to the oxidation of 8.13×10^{-2} μmole of uric acid per minute. Specific activity is defined as the number of enzyme units per mg. of protein, as measured by the use of the biuret reaction (16) with crystalline bovine plasma albumin (Armour) as a primary protein standard. The concentration of protein determined in this manner for an enzyme of highest purity attainable is in satisfactory agreement ($\leq$10 per cent variance) with the value determined from the absorbancy at the peak in the ultraviolet (17).

Metal Analyses; Treatment of Samples—For metal analyses of samples, all chemical operations, dialyses, etc., were carried out with glass-distilled water and glassware which had been rinsed in order with deionized water, glass-distilled water, 0.001 M Tris Versenate, and finally with a solution of dithizone in carbon tetrachloride. For spectrographic analyses the samples were dialyzed first against 0.01 M carbonate or phosphate, pH 10.5, containing 0.001 M disodium Versenate for 12 hours. This was followed by dialysis against 0.01 M Tris Versenate, pH 8.1, for 24 to 48 hours. The enzyme precipitated under these conditions. The preparation was transferred to thick walled glass centrifuge tubes and spun at top speed in the high speed head of the International centrifuge for 10 minutes. The supernatant buffer solution was then poured off and the bulk of the remaining fluid removed by inverting the tubes over filter paper and allowing them to drain. Final drying was achieved *in vacuo* over P_2O_5.

For quantitative chemical analyses and determination of activity, the

dialysis procedure was reversed. The enzyme was first precipitated by dialysis against Tris Versenate, pH 8.1, followed by a change in the dialysis medium to the phosphate-Versenate or the carbonate-Versenate buffer; the enzyme redissolves under these conditions.

Qualitative Metal Analyses—Qualitative analyses were performed by arc spectroscopy in a manner described previously (18). Duplicate samples were submitted to the Department of Chemistry, University of Wisconsin,[3] and to a commercial organization.[4] The blank in each case consisted of an equal volume of dialysis buffer treated in a manner entirely analogous to the sample.

Quantitative Copper Analyses—The method used was a micromodification of the dithizone procedure (19), involving a wet digestion of the sample with concentrated H_2SO_4, followed by a digestion with H_2SO_4 and H_2O_2. For greatest precision we have carried a known amount of a standard solution of copper through the digestion procedure, both in the presence and in the absence of the sample. In this manner we have obtained adequate checks on the amount and reproducibility of the color yield due to a known amount of standard under the exact conditions of each individual determination. This procedure eliminates non-specific effects due to the presence of impurities and variation in the base-line absorption of reagents. The value for each experimental determination was corrected for a blank obtained by determination of the copper content in a volume of dialysis fluid equal to that in which the sample was contained.

Isolation and Purification of Enzyme

The method of isolation takes advantage of the fact that uricase is associated with the mitochondrial fraction of liver cells (20). Pork liver mitochondria are prepared as previously described (21) and are then subjected to acetone drying. Essentially the same number of total units is obtained if an alkaline extract of an acetone powder of whole liver is compared with a similar extract of acetone-dried particles, although the total protein concentration is considerably lower in the latter case.

The further purification stages, given in Table I, make use of the fact that uricase is very stable and particularly susceptible to isoelectric precipitation under a variety of experimental conditions. All operations are carried out at 0–2°, unless otherwise indicated. Some of the steps are similar to those used by Oppenheimer and Kunkel (22) and by Leone (7) in their purification procedures.

Extraction of Acetone Powder—100 gm. of fresh acetone powder of pig mitochondria are suspended in 1 liter of 0.1 M phosphate, pH 7.8, and

[3] We are indebted to Professor V. W. Meloche for performing these analyses.

[4] American Spectrographic Laboratories, San Francisco, California.

stirred vigorously for 30 minutes at 0°. At the end of this period the extract of soluble protein is separated from the residue by centrifugation at the highest *g* value practicable, and discarded. This supernatant fluid contains essentially no uricase, but between 75 and 80 per cent of the total protein of the powder. The residue, freed as much as possible from adhering buffer, is resuspended in 1000 ml. of 0.15 per cent Na_2CO_3 and stirred vigorously for 30 minutes. The suspension is freed of particles by high speed centrifugation; the supernatant fluid contains essentially all the uricase activity. (Fraction A, specific activity, 0.075 to 0.2 unit per mg.; total activity, 600 to 1200 units.)

Precipitation with Ammonium Sulfate and Heat Treatment—To Fraction A (600 ml.), 95 ml. of an alkaline (pH 8.5)-saturated ammonium sulfate solution are added slowly with stirring. The resulting suspension is centrifuged for 20 minutes at full speed in the No. 845 head of the International centrifuge, and the residue discarded. To the supernatant fluid are added 450 ml. of the ammonium sulfate solution, and the mixture is again centrifuged for 30 minutes. The residue is freed carefully of the adhering salt solution and is finely suspended in 180 ml. of distilled water by means of a Potter-Elvehjem homogenizer. The temperature of the mixture is raised quickly to 55–60° by means of a water bath and is maintained in this range for exactly 5 minutes. The suspension is rapidly chilled to 0° and then centrifuged for 10 to 15 minutes. The residue is discarded; the clean yellow supernatant fluid constitutes Fraction B (500 to 1000 units, specific activity, 0.40 to 1.0).

TABLE I

Purification of Typical Uricase Preparation

Fraction	Description	Total volume	Total protein	Total units	Specific activity	Over-all recovery
		ml.	*mg.*		*units per mg.*	*per cent*
A	Carbonate extract	600	5550	1170	0.21	100
B	Heat-treated supernatant	200	760	730	0.96	63
C	Ammonium sulfate ppt.	17	127	700	5.5	60
E	*tert*-Butanol extract	3.5	21	500	23.8	43
F	Tris Versenate ppt.	2.0	6.6	400	60	34
G	Alkaline ammonium sulfate	2.0	2.0	250	125	21

Treatment with Calcium Phosphate Gel and Precipitation with Acid Ammonium Sulfate—To 220 ml. of Fraction B are added dropwise 44 ml. of tricalcium phosphate gel (well aged, about 20 mg. of dry matter per ml.). The gel is removed by centrifugation, and to the supernatant fluid (256 ml.) are added, in small increments, with constant stirring, 40 gm. of

solid ammonium sulfate. The mixture is then allowed to stand, again with stirring, for an additional 20 minutes, centrifuged for 20 minutes at full speed, and the residue dissolved in the minimal volume of 0.1 M Na_2CO_3 (10 to 20 ml. will be necessary). This solution constitutes Fraction C (400 to 800 units, specific activity, 2.0 to 8.0).

Dialysis against Bicarbonate—Fraction C is dialyzed against 50 volumes of 0.02 M $KHCO_3$ for 12 hours, followed by dialysis against a second batch of the same amount of the same buffer. The enzyme precipitates under these conditions. It is collected by high speed centrifugation, and suspended by homogenization in 2.0 ml. of 1 per cent Na_2CO_3. (Fraction D, specific activity 10 to 20 units per mg.; total activity 400 to 750 units.)

Solubility with tert-Butyl Alcohol—To Fraction D is added 0.25 ml. of a mixture of *tert*-butyl alcohol (60 per cent)-water (40 per cent), and the preparation is allowed to stand at 0° with occasional stirring for 4 to 6 hours. It is then frozen, stored at −12° overnight, thawed, and centrifuged at high speed. The enzyme is recovered almost quantitatively in the supernatant fluid (Fraction E, specific activity, 20 to 40 units per mg.; total activity, about 400 to 500 units). Until this point, spectra of the enzyme show a higher extinction at 260 than at 280 mμ, but after this step the E_{260} drops abruptly and subsequent purification steps increase the value of $E_{280}:E_{260}$ even further. This has been interpreted to mean that crude uricase preparations are associated with nucleic acid or nucleic acid breakdown products, and many of the apparent anomalies of uricase preparations described in the literature may be explained in this manner.

Precipitation with Tris Versenate—Fraction E is dialyzed for 18 to 24 hours against 0.01 M Tris Versenate, pH 8.0 to 8.1. The enzyme precipitated in this manner is collected by high speed centrifugation and is then redissolved in 1 per cent Na_2CO_3 or in 0.02 to 0.1 M phosphate buffer, pH 10.5. About 50 to 60 per cent of the activity originally present in Fraction E is found in this solution (Fraction F, specific activity, 40 to 70 units per mg.; total activity, 250 to 450 units).

Fractionation with Alkaline Ammonium Sulfate—A solution of saturated alkaline (ammoniacal) ammonium sulfate is added dropwise with stirring to Fraction F. Four fractions precipitating with increasing salt concentrations are collected serially. After centrifugation, the fractions are dissolved in alkaline phosphate or carbonate, and assayed for enzymatic activity. The highest specific activity is generally found in one or both of the two center fractions, precipitating usually at an ammonium sulfate concentration around 30 to 35 per cent of saturation. (Fraction G, specific activity, 100 to 125 units per mg.; total activity, 150 to 275 units.)

Preparations of specific activity 120 to 125 cannot be purified further by repetition of any of the above purification steps either singly or in combination, by fractionation with salts either at alkaline or neutral pH,

by further isoelectric precipitation, by fractionation with alcohol in the presence or absence of Zn^{++}, or by adsorption on and elution from calcium phosphate, alumina $C\gamma$, or zinc hydroxide gels.

Purification and Recovery—The method described leads to an over-all purification of some 1000-fold over the first alkaline mitochondrial extract, or some 4000- to 5000-fold over the total extractable proteins from acetone-dried mitochondria. The over-all recoveries observed ranged from 30 to 80 per cent to the stage of Fraction E, and from 10 to 30 per cent to the stage of Fraction G.

Properties of Purified Enzyme

Examination in Ultracentrifuge[5]—A preparation with a specific activity of 120 was examined in the analytical ultracentrifuge with a rotor speed of 53,000 r.p.m. Under the conditions of the experiment, *i.e.* a 0.2 per cent protein solution in 0.1 M phosphate buffer, pH 10.5, at 22.5°, only one sharp, symmetrical boundary was observed. The sedimentation constant, corrected to water and 20° ($s_{20,w}$), was calculated to equal 5.5×10^{-13} sec. Assuming a partial specific volume of 0.74 ± 0.01 and a frictional ratio of 1.30 ± 0.15, this would correspond to a molecular weight of approximately 1×10^5.

Examination on Electrophoresis[5]—After preliminary dialysis against 0.1 M phosphate buffer, pH 10.5, a sample (specific activity, 120) was examined in a 4 ml. electrophoresis cell in the same buffer, with a 3 ma. current. After a total migration time of 120 minutes, only one sharp, symmetrical boundary could be detected. The mobility was calculated to equal 3.8×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$.

Appearance and Stability of Enzyme—Solutions of the enzyme, even those of fairly high concentration, are completely transparent and show no trace of color. At pH values greater than 9.8 to 10, the enzyme is soluble in buffers of ionic strength 0.05 to 0.20 up to a protein concentration of 1.5 per cent, the highest value tested. In a 0.1 M phosphate buffer of pH 10.5 or a 1 per cent Na_2CO_3 solution, the enzyme remained perfectly soluble up to 100 hours at 2°, the longest time tested, and full activity was maintained throughout this period. No impairment in enzymatic activity was observed by exposure of the enzyme to hydrogen ion concentrations corresponding to pH values ranging from 1.0 to 12.5 for 10 minutes at 0°. Similarly, full catalytic efficiency is maintained even in dilute aqueous, or aqueous, buffer solutions down to a protein concen-

[5] We are indebted to Professor R. M. Bock for carrying out these determinations. In the electrophoresis experiment the boundary appeared to migrate as a steady state spike. This criterion for homogeneity has been described and discussed by Hoch (23) and Anderson (24).

tration of 0.01 per cent on storage at 2° overnight or at −12° in the frozen state for several days. Repeated freezing and thawing also do not seem to impair the activity of the preparation. If the enzyme is kept frozen for periods greater than 2 weeks, a gradual decrease in activity can be observed.

Activity and Turnover Number—Assuming a molecular weight of 100,000, a specific activity of 125 corresponds to a turnover number of 1000 moles of uric acid oxidized per mole of enzyme per minute at 22°, at pH 8.0, in water in equilibrium with air and at a uric acid concentration of 4×10^{-5} M. By using the factors to be reported subsequently to correct for a temperature of 38°, an oxygen atmosphere, and limiting uric acid concentration, we find a turnover number of $1000 \times 2.6 \times 1.8 \times 1.7 = 8000$.[6] If the reaction were to be run at the optimal pH of 9.0, this value would be raised to 12,000.

A turnover number of 8000 corresponds to a Q_{O_2} (protein) of 117,500. Experimentally, 4.0 γ of enzyme were observed to catalyze the uptake of 36 c.mm. of O_2 in 10 minutes at 38° (gas phase, O_2, uric acid concentration 1×10^{-4} M, 0.02 M borate buffer, pH 8.0), corresponding to a Q_{O_2} of 120,000.

Identification of Cu As Prosthetic Metal of Enzyme

Experiments with Complex-Forming Agents—In accord with previous investigators, we believed that the rapid, reversible, and efficient inhibition of uricase action by cyanide ions (2) provided a clue as to the nature of the prosthetic group of the enzyme. Cyanide can function as a reagent for binding either a prosthetic metal or a carbonyl group. To investigate the second possibility, a series of carbonyl reagents was tested under conditions of pH and incubation time corresponding to those obtaining for cyanide inhibition, but at concentrations exceeding that of cyanide by at least a factor of 10. These data are summarized in Table II. It can be seen that, even under these favorable conditions, carbonyl reagents are incapable of blocking uricase action effectively.

The metal-binding capacities of cyanide may manifest themselves in two ways, either by actual removal of the metal from the enzyme and binding of the cation as an inorganic cyano complex or by forming a cyano complex with the enzyme-bound metal. The former mechanism, exemplified by Kubowitz' experiments on phenoloxidase (25) and our own on butyryl CoA dehydrogenase (18), can be ruled out in the present case because (*a*) cyanide inhibition can be overcome by dialysis of the cyanide-treated

[6] The rate at 38° is approximately 2.6 times that at 22°; at 38° the rate in pure O_2 is 1.8 times that in air; the extrapolated rate at infinite uric acid concentration ($v_{max.}$) is 1.7 times that observed under standard assay conditions.

enzyme and because (*b*) prolonged dialysis against cyanide of a given concentration does not lead to more complete inhibition when an enzyme treated in this manner is compared with one exposed only briefly to the inhibitor and tested immediately (see also Table VI, Experiment F). Thus cyanide inhibition might be interpreted in the present instance as being due to the blocking of an enzyme-linked metal ion by direct combination. In this case cyanide inhibition should not be unique, and the effect should be capable of duplication by other strong metal-binding agents. Accordingly we studied the effect of metal complex-forming and

TABLE II

Effect of Carbonyl and SH— Reagents on Uricase

Inhibitor	Concentration	Per cent inhibition
	M	
Cyanide	5×10^{-5}	98
	1×10^{-5}	76
	5×10^{-6}	45
	1×10^{-6}	8
Bisulfite	5×10^{-7}	5
	1×10^{-3}	10
Hydroxylamine	2×10^{-3}	0
Semicarbazide	1×10^{-3}	11
Phenylhydrazine	1×10^{-3}	5
p-Chloromercuribenzoate	2.5×10^{-4}	5
Iodosobenzoate	1×10^{-3}	0
Arsenite	1×10^{-3}	−10

In all the experiments, the inhibitors were added to the enzyme (3.5 γ of specific activity, 38) and buffer in the standard assay system and incubated for 5 minutes at 22°. The reaction was started by the addition of uric acid (20 γ).

chelating agents, as shown in Table III, where the results of a large series of experiments are summarized. In agreement with previous findings (2, 4, 5), strong complex-forming agents such as fluoride, thiocyanate, and azide, and chelating agents such as 8-hydroxyquinoline, the various Versenes, and diethyl dithiocarbamate are all without inhibitory effect on the enzyme under the conditions of the experiment.

In reexamining the possible mode of action of cyanide in the present case, we became cognizant of the possibility that cyanide inhibition might involve not the oxidized but the reduced form of a metal enzyme and that cyanide might act both as a reducing agent and as a complex former. Accordingly we cast about for other reagents capable of both these functions. As seen in Table IV, ascorbate and hydroxylamine, both effective reducing agents and efficient binders of heavy metals (especially of iron

and copper), are effective inhibitors of uricase under appropriate conditions; *i.e.*, a period of preincubation. The experiments involving fluoride

TABLE III

Effect of Metal-Binding Agents on Uricase

Reagent	Per cent inhibition*
Thenoyl trifluoroacetone	0
Diethyl dithiocarbamate	−10
EDTA	0
N-Hydroxyethylethylenediaminetriacetic acid	0
8-Hydroxyquinoline	2
Tiron	0
Fluoride	0
Thiocyanate	15
Azide	0

All inhibitors at 10^{-3} M preincubated with the enzyme (2 γ of specific activity of 100 in 0.01 M borate buffer, pH 8.0) for 30 minutes at 30°; reaction was started by the addition of uric acid.

* For a highly purified enzyme (specific activity ≥60), with enzymes of lower specific activity, metal-binding agents frequently appear to stimulate the activity.

TABLE IV

Effect of Hydroxylamine and Ascorbate on Uricase

Reagent	Previous treatment of enzyme	Per cent inhibition
Ascorbate	None	75
Hydroxylamine	"	48
" + fluoride	"	95
" + KCNS	"	73
Sulfide	"	5
Semicarbazide	"	0
CN^- (2.5×10^{-6} M)	"	75
None	8-Hydroxyquinoline	0
"	Diethyl dithiocarbamate	0
Ascorbate	8-Hydroxyquinoline	7
CN^- (2.5×10^{-6} M)	"	5
" (2.5×10^{-5} ")	"	50
Hydroxylamine + fluoride	Diethyl dithiocarbamate	0

All the conditions are similar to those in Table III. The 8-hydroxyquinoline- and diethyl dithiocarbamate-treated enzymes were prepared by dialysis of the preparation against these agents and are described in the text.

are of interest; fluoride alone is completely ineffective as an inhibitor (Table III). Hydroxylamine at the concentration shown leads to approxi-

mately 50 per cent inhibition. This effect is probably due to simultaneous (or closely consecutive) reduction by one hydroxylamine molecule and binding of a second one. Hydroxylamine in the present case is a more effective reducing agent than a metal complex-forming agent. Thus an enzyme prereduced by hydroxylamine is now capable of binding and being inhibited by fluoride. The fact that sulfide, which should be able to perform these two functions, is not a good inhibitor might be explained by steric considerations.[7]

Binding of Chelating Agents by Enzyme—Even though most ordinary chelating agents do not function as inhibitors, they can be shown to be bound by the enzyme. Thus, dialysis of the enzyme against 0.01 M Tris Versenate at pH 8.0, followed by a second period of dialysis against the same buffer containing 0.001 M sodium diethyl dithiocarbamate, leads to an accumulation of a yellow-colored material inside the dialysis bag. Examination of the contents indicates that it is the enzyme which can be shown to have acquired a brilliant yellow color with an absorption spectrum (Fig. 1), which appears to be typical of dilute solutions of Cu diethyl dithiocarbamate complex, either in the presence or in the absence of Cu-binding proteins. No other metal complexes portray a spectrum resembling the one observed, nor even any appreciable absorption in the characteristic region around 450 mμ (Fig. 1). Similar experiments can also be performed with 8-hydroxyquinoline, leading to a green complex. The spectrum of this complex is not as specific, however, and might be due to the presence of any of several different metals.

When the enzymatic activity of these presumptive complexes of a protein-metal-chelating agent is compared with that of the untreated enzyme, the results in the lower half of Table IV are obtained. The complex-forming enzyme is as effective as the untreated variety. This suggests that the substrate might be bound to the enzyme in a manner analogous to that of the chelating agents, and is capable of competing successfully with these reagents for the metallic site on the enzyme. The chelated enzyme preparations do portray a significant difference in their behavior toward the metal-binding inhibitors (cyanide, etc.) discussed in the previous section. Thus, as the data of Table IV indicate, these transformed enzymes are completely protected against ascorbate and hydroxylamine inhibition, and partially protected against cyanide inhibition as well.

Substrate, if bound in the same manner at the same metal site, should then produce similar protective effects. This is borne out by the data in Table V. Preincubation with substrate leads to a decrease in the in-

[7] The addition of sodium sulfide in solution does not provide the ions $S^{=}$ or HS^{-} but polymeric forms also involving neutral sulfur, which might well be too bulky to be able to penetrate the active site on the enzyme.

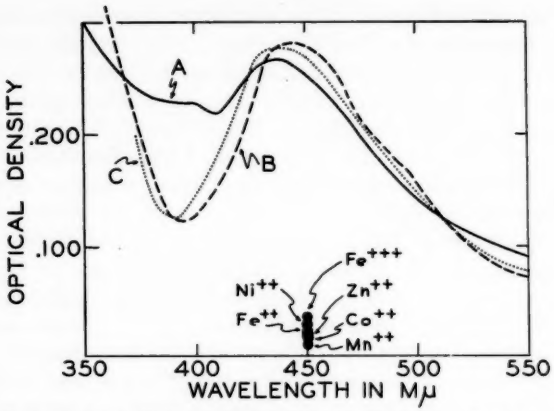


FIG. 1. Spectra of Cu diethyl dithiocarbamate chelates. Curve A, uricase (Cu content, 2 γ per ml., specific activity, 40) dialyzed for 12 hours against 0.01 M Tris Versenate, pH 8.0, followed by dialysis against the same buffer containing 0.001 M sodium diethyl dithiocarbamate for 24 hours. Curve B, 2 γ of Cu^{++} per ml. (as the sulfate) treated for 60 minutes with 0.001 M diethyl dithiocarbamate in 0.01 M Tris Versenate, pH 8.0, at 38°. Curve C, conditions as in Curve B, but 0.93 mg. per ml. of recrystallized bovine albumin or 1.0 mg. per ml. of metal-binding human globulin were added. The solid circles indicate Mn^{++}, Co^{++}, etc., obtained under conditions similar to those used for the data of Curve B, but with the appropriate ion at the same concentration substituted for Cu^{++}.

TABLE V

Effect of Preincubation with Uric Acid on Uricase Inhibited by Metal Complex-Forming Agents

Reagent	Per cent inhibition
Hydroxylamine	54
" + fluoride	95
Ascorbate	98
Hydroxylamine + uric acid	0
" + " " + fluoride	5
Ascorbate + uric acid	5
Cysteine + " "	0
Diethyl dithiocarbamate + uric acid	0
EDTA + uric acid	10
N-Hydroxyethyliminodiacetic acid + uric acid	15
8-Hydroxyquinoline + uric acid	12
Furoyl trifluoroacetone + uric acid	0

20 μmoles of diol buffer, pH 8.5, 300 γ of uric acid (where indicated), 20 γ of enzyme (specific activity, 60), and 0.1 μmole of inhibitor were incubated for 2 hours at 22° in a total volume of 0.10 ml. At the end of this period 0.01 ml. aliquots were withdrawn and tested in the standard assay system.

hibitory efficiency of hydroxylamine and ascorbate. No great enhancement of the inhibitory power of the metal-binding agents of Table III, and of numerous others such as cysteine, *o*-phenanthroline, or —SH inhibitors, is observed under these reducing conditions. Is substrate then bound at the same site as the chelating agents? From the following experiment, this would appear to be the case: A diethyl dithiocarbamate-linked enzyme, well dialyzed against buffer (0.1 M borate, pH 9.0) was made 10^{-4} M with respect to sodium urate, while another aliquot of the same enzyme was made 10^{-4} M with respect to sodium chloride. Dialysis at 0° was then resumed against the same buffer. After 1 hour, the two bags were opened and the E_{450} determined. Since the value of the chloride-

TABLE VI

Copper Content of Purified Uricase Preparations

Preparation	Protein	Cu content	Specific activity	Activity	Mol. wt., 1 atom Cu per mole
	mg. per ml.	*γ per mg.*	*units per mg.*	*units per γ Cu*	
A	3.0	0.45	120	270	145,000
B	3.0	0.67	125	180	97,000
C	2.4	0.22	40	185	87,000
D	4.2	0.071	15	211	73,500
E	2.0	0.18	40	220	108,600
F*	1.05	0.17	40	240	115,000
G	4.0	0.15	40	260	130,500
H	1.4	0.12	16	133	43,000
I	3.1	0.12	35	290	187,000
K	1.5	0.15	37	245	138,000

* Preparation E was dialyzed *versus* 0.01 M cyanide for 48 hours and retested.

containing enzyme was 0.150 and that of the urate-containing enzyme 0.050, a significant dissociation of the chelating agent from the enzyme under the influence of the substrate could be inferred.

Qualitative Metal Analysis—The experiments just described made it appear profitable to consider again a possible rôle of metals in uricase action, with special emphasis on copper, highly purified preparations of the enzyme being used. Therefore two samples of uricase of specific activity 40 and 120, respectively, were subjected to qualitative spectroscopic analysis. The results obtained in two different laboratories were in essential agreement. Copper was present in all the experimental samples. The copper content of the samples corrected for blank values appeared to increase with increasing specific activity when compared with the experimental sample of lower specific activity. Other metals, except iron (but including Mg, Mn, Zn, Co, Ni, Ag, Hg), appeared to be either absent, or

present at the same level in the experimental sample as in the blank. Iron was present only in the sample of low specific activity, absent in that of high activity.

Quantitative Copper Analysis—In order to investigate the possible rôle of copper as an electron carrier in the enzyme, a systematic study of the copper content of numerous enzyme preparations at different purity levels was undertaken. The results of this study are given in Table VI. There appears to be considerable scatter among the results, the cause for which

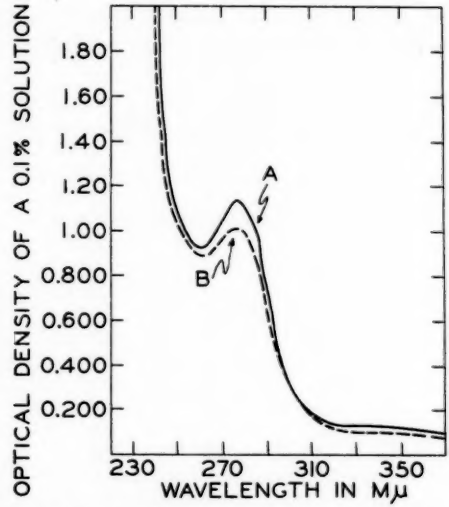


FIG. 2. Spectra of highly purified uricase. Curve A, enzyme of specific activity 120 in 1 per cent carbonate. Curve B, enzyme of specific activity 60.

experimental variation is not apparent at the present time. In spite of these variations, the following facts emerge from Table VI: (*a*) There is an increase in the copper content paralleling an increase in specific activity; (*b*) the characteristic ratio of Cu content per enzyme unit (units per microgram of Cu), which is proportional to the ratio of copper atoms per enzyme molecule, gives a sufficient constancy throughout the purification range (*i.e.*, for enzymes of approximately 10 per cent purity or better) to suggest a definite catalytic rôle for the metal; (*c*) the molecular weight of the enzyme calculated from copper content, assuming 1 copper atom per enzyme molecule, equals 120,000 ± 40,000 in satisfactory agreement with the value calculated from the sedimentation constant.

Absorption Spectrum—Contributory evidence for the hypothesis that

uricase is a copper protein comes from a study of appropriate absorption spectra in the ultraviolet region. The absorption spectra of two highly purified uricase preparations are shown in Fig. 2. Although apparently quite similar to that of a pure protein, not in a complex with any prosthetic group, certain significant differences emerge if the spectrum of the enzyme is compared with that of a pure "typical" protein; *e.g.*, crystalline bovine serum albumin or the metal-binding globulin of human plasma (26). When the latter two proteins are incubated with a Cu^{++} solution, and their spectra redetermined with the metal solution as the blank in this determination at least a qualitative similarity between these copper protein spectra and that of uricase may be observed. This similarity becomes

TABLE VII

Optical Properties of Various Copper-Containing Proteins

Protein	$\lambda_{max.}$	$\lambda_{min.}$	$E_{280}:E_{330}$
Uricase (specific activity 55)	277	261	9.5
" (" " 120)	276	261	5.55
Bovine plasma albumin	280	265	17.6
" " " + Cu	282	275	3.8
Metal-binding globulin	282	265	27.5
" " + Cu	278	270	9.3
" " + Fe	281	265	13.5

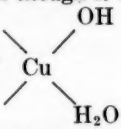
All spectra in 1 per cent Na_2CO_3, with proteins at a concentration of 1.0 mg. per ml., against blanks containing all components except the protein. Spectra in the presence of metal were obtained by incubating the protein in question with 5×10^{-5} M metal ion at room temperature for 45 minutes.

even more apparent when the optical properties of these various proteins are compared as in Table VII. There the most characteristic feature appears to be the ratio of the optical densities at 280 and at 330 mμ. This ratio is high for all "ordinary" proteins and for the iron-globulin complex, but low for all the copper proteins and for uricase.[8]

DISCUSSION

The implication of copper as an integral part of uricase appears somewhat surprising in view of two well substantiated observations reported in the literature (2–5) and fully confirmed in the course of the present investigations. These are that (*a*) diethyl dithiocarbamate, a complex-

[8] The composition and the properties of metal complexes of the metal-binding globulin and of albumin have been studied extensively. These studies have recently been reviewed by Klotz (27). 2 gm. atoms of metal are bound per approximately 100,000 gm. of either protein.

forming agent with a very high affinity for copper, does not inhibit uricase and even enhances the activity of certain preparations, and that (*b*) copper at concentrations of 2×10^{-5} M or above inhibits the enzyme to the extent of 75 per cent or better. The enhancement of activity by diethyl dithiocarbamate is observed only with impure preparations, and never after the dialysis steps in the purification procedure. Thus it is almost certainly related to the second effect. It is conceivable that the enzymatically active site, or one closely adjacent thereto, has a high binding capacity for copper, even though it contains copper itself. Thus a site containing the structure *E*  would be capable of binding an additional Cu ion or some other cation of similar steric requirements in a manner analogous to that found in the inorganic hydrous metal gels and the metal hydroxides. Mn^{++}, an ion of dimensions similar to Cu^{++}, is a good inhibitor of uricase (50 per cent inhibition at 5×10^{-5} M).[9] The rôle of copper receives support from the observations of Griffiths (28) on the non-enzymatic air oxidation of urate catalyzed by Cu^{++} ions. It will be shown in a subsequent paper that this catalysis is exceedingly specific for copper and that optimal interaction rates are obtained only between urate and copper complexes of appropriate structure.

SUMMARY

Uricase has been obtained in a highly purified form by extraction of acetone-dried pork liver mitochondria with alkaline buffer, followed by a series of fractionations under a variety of conditions. It is a colorless protein, soluble in alkaline buffers, with a molecular weight of about 10^6. The enzyme of highest purity shows a Q_{O_2} of 120,000 corresponding to a turnover number of 8000 at pH 8.0 and 38°. Enzymatic activity appears to be related to the presence of copper in the enzyme, the purest enzyme having a copper content of approximately 0.05 weight per cent. The copper is bound by a variety of agents, but only simultaneous reduction and binding lead to efficient inhibition by such agents as cyanide, hydroxylamine, and ascorbate. Binding of chelating agents incapable of reducing the enzyme and of substrate appears to occur on the same metallic site.

[9] In spite of repeated and extensive efforts, it has not been possible so far to reduce the copper content of the enzyme by dialysis or treatment of the enzyme under extremes of pH or high concentrations of metal-binding agents in a reproducible manner; nor have we been successful in reproducibly reactivating preparations of low copper content by incubation with cupric ion.

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A FLAME PHOTOMETRIC METHOD FOR THE DETERMINATION OF PLASMA MAGNESIUM AFTER HYDROXYQUINOLINE PRECIPITATION*

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The numerous methods for determining magnesium in biological fluids that have been reported in the past have varied from chemical to spectrochemical techniques. Among these various procedures have been included gravimetric, colorimetric, and titrimetric techniques.

The gravimetric micromethod based on the precipitation of magnesium 8-hydroxyquinoline in hot ammoniacal solution, first utilized by Hahn (1) in 1926, has been implemented by numerous colorimetric applications. The hydroxyquinoline has been determined colorimetrically in the method of Yoshimatsu (2) and of Eichholtz and Berg (3) by utilization of the Folin phenol reagent. A further colorimetric application of the hydroxyquinoline precipitation involving the formation of a specific green-blue color by the reaction of ferric ions with magnesium hydroxyquinoline has been described by Hoffman (4). He does not consider the possible complications encountered in hemolyzed serum samples.

Independent of the hydroxyquinoline precipitation, some of the more recent colorimetric procedures have utilized the well known titan yellow reaction (5, 6). Essentially, titrimetric methods have involved bromination of magnesium hydroxyquinoline with bromate and eventual iodometric titration of the excess bromate. Greenberg and Mackey (7) have described such a procedure, introducing the Kirk-Schmidt micro filter (8) for collection of the precipitate. Further modifications of the bromination technique have been described by Velluz (9), Berg (10), and Glomaud (11).

The variety of methods described and the numerous modifications of each suggest a general dissatisfaction with them. The need for a sensitive accurate method of magnesium analysis for the determination of low concentrations in biological fluids has suggested the use of flame photometry described here.

By this method, a series of twenty-four determinations of plasma magnesium may be completed in a period of 3 hours.

* Published with the approval of the Chief, Medical Director, of the Veterans Administration. Statements and conclusions published by the author are a result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

Method

Reagents and Equipment—

1. Beckman DU flame photometer with photomultiplier attachment. Oxyhydrogen flame accessories.

2. Micro filter sticks with a sintered glass plug (medium porosity) at the tip, 1 mm. in diameter (supplied by the Microchemical Specialties Company, Berkeley, California). A thin coating of acid-washed asbestos is applied to the tip by suction from a suspension of asbestos in water solution previous to use.

3. 10 per cent trichloroacetic acid (Mallinckrodt, reagent grade).

4. 95 per cent ethyl alcohol.

5. 6 per cent 8-hydroxyquinoline (Mallinckrodt, reagent grade) in 95 per cent ethanol.

6. Acetone-acetic acid solution; 90 per cent acetone-10 per cent glacial acetic acid (by volume).

7. 15 ml. graduated Pyrex centrifuge tubes.

8. Ammonium hydroxide, approximately 58 per cent (Mallinckrodt, reagent grade).

9. 50 per cent ethanol solution saturated with magnesium hydroxyquinoline. 500 ml. of a solution containing about 10 mg. of magnesium per 100 ml. are mixed with 5 ml. of 6 per cent hydroxyquinoline in 95 per cent ethanol. Concentrated ammonia is slowly added until the solution is strongly alkaline. The mixture is heated to boiling. The precipitate of magnesium hydroxyquinoline is removed by filtration with a sintered glass filter and washed three times with distilled water and finally with 95 per cent ethanol. The precipitate is shaken with a liter of 50 per cent ethanol and kept in a dark bottle. A small portion is filtered off before use.

10. Stock magnesium standard (20 m.eq. per liter) 0.4032 gm. of magnesium oxide (Mallinckrodt, reagent grade), previously heated at 800–900° for several hours, dissolved in 10 ml. of concentrated HCl, and diluted to 1 liter with distilled water in a volumetric flask.

11. Glass stirring rods, 2 mm. in diameter.

12. 0.3 per cent neutral sodium oxalate.

Procedure

Enough neutral sodium oxalate is used so that the blood samples contain no more than 0.3 per cent oxalate; the blood samples are collected, and clear plasma is separated by centrifugation. 1 volume of oxalated plasma is added to 2 volumes of distilled water. Then 2 volumes of 10 per cent trichloroacetic acid are added slowly with shaking to coagulate the protein. The solution is allowed to stand 20 minutes. Protein is

removed by centrifugation, providing sufficient filtrate for the remainder of the determination. A 5 ml. aliquot of the clear filtrate (representing 1 ml. of the original plasma) is pipetted into a 15 ml. graduated centrifuge tube. 0.1 ml. of 6 per cent 8-hydroxyquinoline is added along with 0.2 ml. of concentrated ammonium hydroxide. After mixing the contents with stirring rods, the tubes containing the stirring rods are placed in a 75° water bath for 40 minutes. At this time, a series of working magnesium standards varying from 0.5 to 5 m.eq. per liter is prepared from the stock magnesium standard (20 m.eq. per liter). 2.5, 5.0, 10, 15, 20, and 25 ml. of stock magnesium standard representing, respectively, 0.5, 1, 2, 3, 4, 5 m.eq. per liter of magnesium are pipetted into each of six 100 ml. volumetric flasks. 40 ml. of 10 per cent trichloroacetic acid are added to each volumetric flask, and all are diluted to 100 ml. with distilled water and mixed.

1 ml. of each working standard is added to a 15 ml. graduated centrifuge tube along with 0.1 ml. of 6 per cent 8-hydroxyquinoline and 0.2 ml. of concentrated ammonium hydroxide. Finally 3 mg. of sodium oxalate are added. After thorough mixing with a stirring rod, the tubes, still containing the rods, are placed in a 75° water bath for 40 minutes. From this point, both plasma filtrates and standards are treated in the same manner.

After the 40 minute heating period, the stirring rods are carefully washed off with 95 per cent ethanol to form a layer of alcohol about 1 cm. high over the solution. The tubes are capped with Parafilm and centrifuged. The supernatant solution is aspirated with the asbestos-covered micro filter stick and suction from a vacuum line or a water aspirator pump. Care is taken not to disturb the precipitate packed at the bottom of the centrifuge tube. Each filter stick is suspended from a test-tube rack in such manner that the tip end of the filter stick hangs freely.

The precipitate in each tube is washed twice thoroughly with 3 ml. of the 50 per cent ethanol solution, saturated with magnesium hydroxyquinoline. After each washing, the precipitate is packed down by centrifugation, and the supernatant fluid is aspirated with individual filter sticks. After completion of the washing process, each filter stick is placed in its respective centrifuge tube, the sintered glass end resting in the tube. The tubes are then placed in an oven at 130° until dry. A period of 1 hour usually is necessary.

At the end of the drying period, the tubes and sticks are cooled to room temperature. 5 ml. of the acetone-acetic acid mixture are added to all tubes. The precipitates are dissolved by stirring with the filter sticks. After complete mixture, the filter sticks are removed, and the tubes are capped with Parafilm and centrifuged. All samples, including the standards for the calibration curve, are now ready for flame photometry. With

the Beckman DU oxyhydrogen flame photometer and photomultiplier attachment, the following settings are found satisfactory: oxygen gas pressure, 20 pounds per sq. in.; hydrogen gas pressure, 3 pounds per sq. in.; wave-length, 285.2 mμ; photomultiplier sensitivity, 4; zero suppression, 1; slit width, 0.08 mm.

Determinations are run with the phototube shutter open at all times, as described by Kingsley and Schaffert (12). Zero adjustment is made with the dark current knob. The blank solution for zero adjustment is the mixed solvent (90 per cent acetone-10 per cent acetic acid). The per cent transmission values of the various magnesium standards are plotted against the respective concentrations as milliequivalents per liter as a steep smooth curve. The concentration of each plasma sample is estimated by reference to the curve obtained with the use of simultaneous standards.

EXPERIMENTAL

Methods involving the development of analytical procedures for sodium, potassium, and calcium in biological fluids by flame photometric methods have not been appreciably hampered by low detection limits. As indicated in a recent bulletin by Beckman Instruments, Inc., when the model DU with oxyhydrogen flame and photomultiplier attachment is employed, the detection limits for sodium, potassium, calcium, is magnesium, in parts per million, are as follows: Na 0.0002, K 0.001, Ca 0.003, Mg 0.2.

The high emission characteristic of sodium, potassium, and calcium favors determination of these elements in biological fluids by direct dilution of the sample. The necessity of separation to eliminate interference with flame spectra is circumvented by a simple dilution process. However, in order to determine magnesium in body fluids, one is hampered by the presence of these other high light-emitting constituents, with the added disadvantage of dealing with an element present in relatively low concentration. A previous attempt to determine magnesium in blood plasma in the presence of these other cations was unsuccessful, owing to the variable background effect of these ions.

In the method developed here, the necessity was recognized of separating magnesium from sodium, potassium, and calcium to analyze magnesium satisfactorily. Magnesium is separated quantitatively as hydroxyquinolate from blood plasma with sufficient washings to remove adsorbed sodium and potassium from the precipitate. Analysis of the washed precipitate for sodium compared with standards by flame photometry indicated that less than 0.02 m.eq. per liter was present. At this concentration of sodium, no effect upon flame photometric readings of magnesium samples was observed. The complete absence of potassium in the magnesium hydroxyquinolate precipitate was demonstrated by the inability to demon-

strate a maximal shift of the meter needle to the left while the wavelength dial moved in the region for potassium determinations (766 mμ) at wide slit openings up to 0.3 mm. Since it was observed that calcium affected the transmittancy values for magnesium (see Fig. 1), the calcium was effectively removed by the use of sodium oxalate in the collection of whole blood. Calcium can be precipitated as a hydroxyquinolate under the same conditions as for magnesium precipitation.

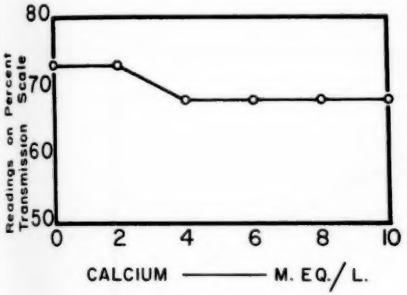

FIG. 1

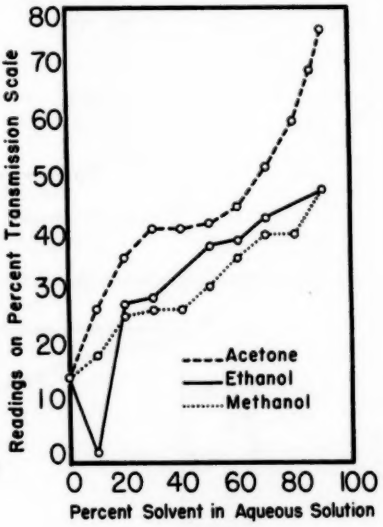

FIG. 2

FIG. 1. Effect of calcium on readings at 285.2 mμ with 2 m.eq. of Mg per liter
FIG. 2. Readings at 285.2 mμ with 2 m.eq. of Mg per liter in different solvents

In order to increase the sensitivity of this procedure, use was made of Kingsley and Schaffert's (13, 14) observation that detection limits in flame photometry can be considerably increased by substituting acetone for water as the solvent.

Fig. 2 demonstrates the relative enhancement of various solvents in per cent transmission for magnesium samples. Of the four solvents, water, methanol, ethanol, and acetone, maximal emission of light is obtained in acetone solution.

Since the magnesium hydroxyquinolate is relatively insoluble in acetone, a minimal amount of acid is required to dissolve the precipitate. Solubility studies indicated that the minimal amount was 4.8 per cent by volume of glacial acetic acid. To attain maximal light emission for each

change in conditions, the effect of glacial acetic in acetone upon per cent transmission values was observed (see Fig. 3). Maximal values were obtained in a mixture of 90 per cent acetone-10 per cent glacial acetic acid. This was the mixed solvent of choice for solution of the magnesium hydroxyquinolate.

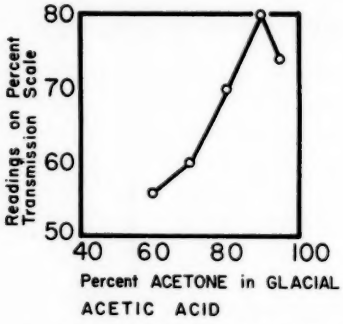

FIG. 3. Effect of acetone-glacial acetic acid mixtures on readings at 285.2 mμ

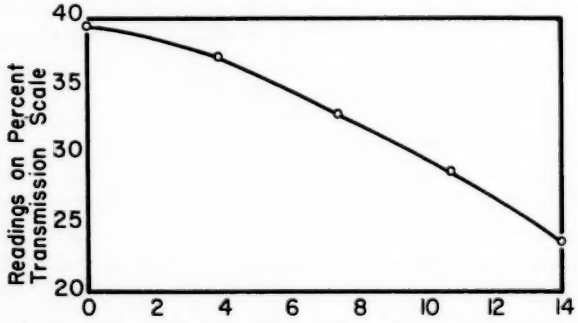

FIG. 4. Effect of addition of water to a mixture of 90 per cent acetone and 10 per cent acetic acid (by volume) on the readings at 285.2 mμ with 2 m.eq. of Mg per liter. Ordinate, volumes of water added.

It is interesting to note that the presence of water while the sample is being burned in the flame photometer will produce marked depression in the per cent transmission (see Fig. 4). The wave-length of choice for the analysis is 285.2 mμ. A 4-fold increase in per cent transmission at this wave-length compared to that at 383 mμ was the determining factor in this choice.

The use of Kirk and Schmidt micro filters was found to be cumbersome and time-consuming in the author's hands. Rapid, satisfactory results in

separation of the precipitate were obtained by precipitation of magnesium in 15 ml. centrifuge tubes and aspiration of the supernatant fluid and washings with micro sintered glass filter sticks. In addition, the filter sticks are used as stirring rods in the final step of the solution of the precipitate.

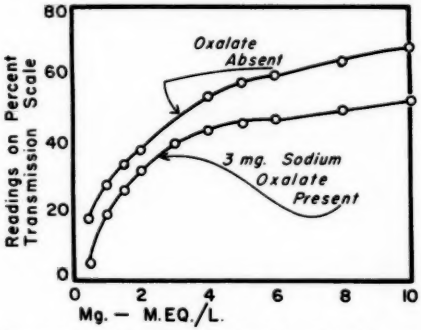

FIG. 5. Calibration curves of Mg standards with and without addition of sodium oxalate at 285.2 mμ.

TABLE I

Recovery of Magnesium Added to Plasma Filtrates

Plasma Mg	Added	Found	Recovery
m.eq. per l.	*m.eq. per l.*	*m.eq. per l.*	*per cent*
1.67	2.0	3.76	102.5
1.92	1.0	2.99	102.3
1.92	1.5	3.51	102.6
1.67	2.0	3.64	99.3
1.67	4.0	5.57	98.4
1.92	2.0	3.89	99.4
2.22	0.2	2.42	100.0
1.29	0.35	1.59	97.0
2.28	0.4	2.64	98.7
1.65	0.58	2.16	97.0

Fig. 5 indicates the effect of oxalate upon the precipitation of magnesium hydroxyquinolate. In attempts to evaluate the effect of oxalate upon the flame spectra of magnesium, it was noted that oxalate, in the concentration range involved (0.06 per cent), was insoluble in the acetone-glacial acetic acid mixture. The depressant effect of oxalate upon the precipitation is compensated for by using simultaneous standards containing the same amount of sodium oxalate as in the blood plasma samples. The calibration curve is a smooth steep curve up to 5 m.eq. per liter. Be-

yond, from 5 to 10 m.eq. per liter, per cent transmission is a straight line function of concentration.

Good recoveries of magnesium added to plasma filtrates are indicated in Table I. Plasma magnesium determinations were run by the method described and also by the titan yellow procedure of Kunkel *et al.* (5) (see Table II).

TABLE II

Comparison of Plasma Magnesium Determinations by Flame Photometric and Titan Yellow Methods

Titan yellow	Flame photometric
m.eq. per l.	*m.eq. per l.*
1.64	1.68
1.52	1.57
1.29	1.39
1.14	1.22
1.62	1.70
1.15	1.20

SUMMARY

A new, accurate, sensitive flame photometric method for the determination of plasma magnesium is described, in which magnesium is separated from its flame interferences.

The author wishes to thank Mr. John J. Heise, Radioisotope Division, Veterans Administration Hospital, for his technical assistance. The author is indebted to Mr. George P. Cave, Veterans Administration Hospital, Cold Spring Road, Indianapolis, Medical Illustration Department, for preparation of the graphs.

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INCORPORATION OF 4-AMINO-5-IMIDAZOLECARBOXAMIDE-4-C^{13} INTO URIC ACID IN THE NORMAL HUMAN

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The isolation of 4-amino-5-imidazolecarboxamide (AIC) from the culture medium of sulfonamide-inhibited *Escherichia coli* (1) and its subsequent identification (2) were followed by numerous studies implicating this compound in the biosynthesis of purines. In bacterial systems AIC is found to accumulate in the culture medium of aminopterin-inhibited *E. coli* (3) and also in the medium of certain purine-requiring mutants (4). It satisfies the nutritional requirement for purines in other mutants of *E. coli* (5). AIC is incorporated into nucleic acid purines and also into nucleotide adenine of yeast (6). In animal systems AIC is incorporated into uric acid by the pigeon (7), into allantoin and tissue purines by the rat (8), and into tissue purines by the mouse (9). The results of other studies suggest that AIC enters into purine biosynthesis as the ribotide rather than as the free base (10–12).

In the course of a study of pathways of uric acid synthesis in normal subjects and in patients with derangements of purine metabolism, isotopic precursors of uric acid have been prepared and fed to humans. In view of the results of animal experiments cited above and the close structural relationship between AIC and uric acid, AIC has been compared with glycine, previously studied (13), as a precursor of uric acid in man. The present paper reports the results of studies on two normal subjects who received AIC-4-C^{13} by mouth.

EXPERIMENTAL

Materials—AIC was synthesized with C^{13} in position 4 by the method of Shaw and Woolley (14) with minor modifications. Ethyl cyanoacetate was prepared from potassium cyanide-C^{13}, approximately 60 atom per cent excess C^{13} (15). In order to secure a yellow crystalline product in the synthesis of phenylazomalonamamidine ((14), compound III), it was found necessary to maintain the pH at 4 by repeated additions of sodium acetate solution. The AIC ultimately obtained was further purified by removal of colored contaminants with activated charcoal (Darco), crystallization

from aqueous solution as the picrate, and conversion to the hydrochloride (8). Traces of picric acid were removed from the crystalline product by continuous extraction with an ethereal solution of dry HCl. Yield, 48 per cent of theoretical, based on $KC^{13}N$. Melting point[1] of the picrate, 238–240° with decomposition.

$C_4H_7ON_4Cl$

Calculated (corrected for C^{13} content). C 29.8, H 4.3, N 34.4, Cl 21.7
Found. C 29.6, H 4.1, N 33.8, Cl 22.1
C^{13} 13.6 atom % excess; N^{15} 0.014 atom % excess[2]

A solution of AIC-C^{13} hydrochloride in 0.01 M phosphate buffer at pH 7.0 showed maximal absorption at 267 mμ (1), with a molecular extinction coefficient, ϵ, of 11,200 sq. cm. × $mole^{-1}$. Under the same conditions, a non-isotopic preparation, purified further by sublimation, gave a value for ϵ of 11,300.

Previous studies of others (9) have revealed that mice tolerate AIC hydrochloride in single doses of 0.6 gm. per kilo of body weight. We have failed to detect any toxicity at dosage levels up to 1 gm. per kilo, administered intraperitoneally to rats, other than the transient appearance of a pink urine, which gave a negative test for blood with benzidine. This was noted only at the highest dose level tested. No histological abnormalities were found in a rat killed 24 hours after injection of AIC hydrochloride at this dosage.

Graded doses of non-isotopic AIC hydrochloride up to 14 mg. per kilo were administered orally to a normal adult human male. A transient sensation of dryness of the nasopharynx was the only effect, objective or subjective, that was noted. Both experimental subjects reported this same sensation when AIC-C^{13} was ingested.

Glycine-N^{15} was prepared by methods previously described (16, 17). Two batches were employed with N^{15} contents of 58.8 and 62.7 atom per cent excess, but each subject received glycine from a single preparation in repeated studies.

Methods of Isolation and Degradation—Uric acid was isolated from urine according to Geren *et al.* (18) and purified by precipitation from Li_2CO_3 solution by the addition of acetic acid (19).

Samples of uric acid weighing 50 mg. were oxidized with Cl_2 (20) to alloxan, which, after reduction with H_2S (21), was isolated as alloxantin. This product was taken to represent N atoms at positions 1 and 3 of uric acid (22). From the oxidation mixture urea was isolated as the dixanthydryl derivative (23), presumably representing N at positions 7 and 9

[1] The melting points were determined on a Fisher block.

[2] Evidently the commercial procedure used for concentration of C^{13} as KCN also results in some enrichment of N^{15}.

of uric acid. An authentic sample of uric acid-1,3-N^{15} containing 28.3 atom per cent excess N^{15} was degraded by the above procedure. The isotope concentrations detected in the degradation products (Table III) represent 0.02 per cent contamination of urea by N from position 1 or 3.

AIC was isolated from 500 ml. portions of urine by adsorption upon Darco S-51. Two successive treatments, each with 4.5 gm. of Darco at 0° for 2 hours, were employed, and each batch of adsorbent was subsequently extracted twice with 100 ml. of boiling 1 N HCl for 30 seconds. The combined acid extracts were passed through a column, 2.2 × 18 cm., containing 50 gm. of Dowex 50-X12 in the acid form. AIC was eluted from the column with 3 N HCl and appeared in the fraction from 430 to 550 ml. as indicated by the Bratton-Marshall test. This portion of the eluate was evaporated to dryness at reduced pressure under N_2, the residue was dissolved in 2 to 3 ml. of water, and the solution was decolorized with Darco and filtered. To the filtrate was added 0.7 ml. of saturated ethanolic picric acid, and the mixture was refrigerated. The product was twice recrystallized from hot water and AIC hydrochloride was formed from the picrate. The melting point of the picrate was 237–238°, with decomposition. The recovery, as the hydrochloride, was 17 per cent of the AIC detected analytically in the urine.

Creatinine was isolated as the zinc chloride double salt by the method of Benedict (24) from the urine filtrates which were obtained in the course of the uric acid isolation.

Analytical Methods—Uric acid in serum and urine was determined by differential spectrophotometry (25, 26) with purified uricase (27, 28).

AIC concentration in serum and urine was measured by a modified Bratton-Marshall procedure (29, 30). Serum was first freed of protein by treatment with 2 volumes of 10 per cent trichloroacetic acid solution.

Isotope abundances were measured with a 60° Nier type mass spectrometer. Carbon isotope ratios were determined on CO_2 samples, prepared by wet combustion of organic compounds (31). The CO_2 evolved was collected in $Ba(OH)_2$ solution and was regenerated for analysis with $HClO_4$ solution in modified Thunberg tubes. Nitrogen isotope ratios were determined on N_2 gas prepared according to the method of Rittenberg (32). Normal abundance of C^{13} was determined on stock $NaHCO_3$ and of N^{15} on air freed of O_2 (33).

In second experiments on subjects who had previously received N^{15}, the residual isotope enrichment in each urinary product, determined on samples secured during the 10 days preceding the second administration, was taken as normal abundance. 2½ to 3 months elapsed between successive experiments on the same subject, and after this interval N^{15} enrichments in creatinine and in uric acid of urine were not declining perceptibly.

Human Studies—Two normal adult male subjects having no stigmata

or family history of gout were studied. For 10 days prior to and during the experimental periods, their diets were free of meat, fish, and legumes. The major portion of the 70 gm. of protein ingested daily came from milk, eggs, and cheese. The diet was constant, nutritionally adequate for weight maintenance, and was very poor in purines. In Part I of each study, glycine-N^{15}, 100 mg. per kilo, and AIC-4-C^{13} hydrochloride, 14 mg. per kilo, were ingested, mixed with the breakfast milk. In Part II, after a lapse of a period sufficient for elimination of most of the isotope, glycine-N^{15}, at the same dosage, but no AIC, was similarly administered.

All urine was collected and preserved at room temperature under toluene. Each portion, representing a collection period of from 6 to 24 hours, was promptly analyzed for uric acid, AIC, and creatinine (34). Uric acid and creatinine were isolated from each urine sample, and in the experiment

TABLE I
Subjects Studied

	J. E. S., 33 yrs.		L. L., 26 yrs.	
	Part I	Part II	Part I	Part II
Weight on which dosage was based, *kg.*	74.3	70.9	73.5	69.4
Serum uric acid, *mg. per 100 ml.*	4.1		5.0	
Average uric acid in urine, *mg. per 24 hrs.*	513 ± 53*	453 ± 58	405 ± 30	393 ± 20

* Mean ± standard deviation.

on J. E. S., Part I, AIC was also isolated from the first urine collection. The uric acid obtained from the 2nd day's urine, in both parts of each study, was degraded, yielding, as ultimate products, alloxantin and dixanthydryl urea. In the study on subject J. E. S., Part I, serum was analyzed for AIC during the first 8 hours after ingestion. In Table I are given some of the characteristics of the two subjects studied.

RESULTS AND DISCUSSION

Fate of Ingested AIC—The ingestion of approximately 1 gm. of AIC by normal adult male subjects was followed, in the succeeding 8 hours, by the urinary excretion of about 20 per cent of the ingested dose, as determined by the Bratton-Marshall test (Table II). The quantity detected was not appreciably increased by acid hydrolysis of the urine prior to analysis, and essentially no diazotizable amine was recovered in the urine after the 14th hour. The serum level of AIC rose in one subject (J. E. S.) to a maximum of 0.9 mg. per 100 ml. at 2 hours and fell to 0.1 mg. by the

8th hour. Of the C^{13} fed as AIC, 20 to 23 per cent was recovered in urinary uric acid in the succeeding 2 to 3 weeks. This leaves approximately 55 to 60 per cent of the administered AIC unaccounted for. On the basis of observations in animals (8), a portion of this remainder is presumed to have entered tissue purines and possibly other metabolites (9).

AIC was isolated from the urine of one subject (J. E. S.) and was found to contain 13.7 atom per cent excess C^{13} as compared with 13.6 in the material fed. Despite the simultaneous ingestion of glycine-N^{15}, the AIC recovered from the urine showed no significant enrichment with N^{15} above

TABLE II

Recovery of C^{13} and N^{15} in Urinary Products

The cumulative recoveries of C^{13} in urinary AIC and uric acid have been calculated as percentages of C^{13} fed as AIC. Similar data are given for N^{15} in urinary uric acid as percentages of N^{15} fed as glycine. In Part I of each study, AIC-C^{13} and glycine-N^{15} were fed; in Part II, only glycine-N^{15} was fed.

	Per cent of administered dose of isotope			
	J. E. S.		L. L.	
	Part I	Part II	Part I	Part II
C^{13} in AIC excreted	20		21	
" " uric acid excreted in 14 days	21		20	
" " " " " " 15–21 days			3	
Total C^{13} accounted for	41		44	
N^{15} in uric acid excreted in 14 days	0.16	0.32	0.09	0.27

that found in the synthetic AIC-C^{13}. From these findings it is concluded that no significant dilution of AIC, orally administered, occurred prior to its urinary excretion. Thus the occurrence of AIC as a normal metabolic intermediate has not been detected in the present experiment. That AIC as such is not a normal intermediate in purine synthesis in various systems studied has been reported by others (8), although its ribotide has been implicated (10–12).

Conversion of AIC to Uric Acid—Though probably not a normal intermediate, AIC is none the less promptly converted into uric acid. Roughly 200 mg. of uric acid in excess of the basal excretion were recovered, in each experiment, in the 2 days following AIC ingestion. The C^{13} concentration in urinary uric acid rose rapidly to a maximum of about 1.3 atom per cent excess (see Fig. 1) at 14 hours. This peak enrichment, in view of the additional carbon atom at position 2 of uric acid, corresponds to an isotope dilu-

tion of 8.5-fold. The dilution is not much greater than would be anticipated if some 20 per cent of the ingested AIC were promptly converted to uric acid and introduced into a miscible pool of uric acid of normal dimensions (35).

The rapidity of conversion of ingested AIC into uric acid is in contrast to the slower rate of conversion of glycine to uric acid. After the feeding of glycine-N^{15}, in previous studies (13) as well as in the present experiments (Part II), approximately 3 to 4 days elapsed before urinary uric acid was maximally enriched with N^{15}.

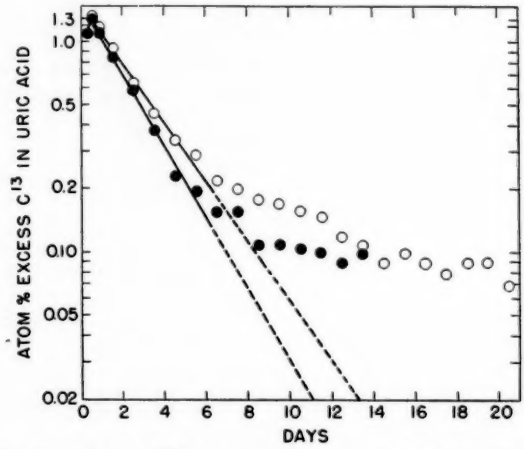

FIG. 1. The abundance of C^{13}, as atom per cent excess plotted hemilogarithmically, in urinary uric acid after feeding AIC-C^{13} to two normal subjects. ●, J. E. S.; ○, L. L.

From the curves in Fig. 1, it is apparent that the decline in C^{13} enrichment of uric acid does not follow first order kinetics. Each curve appears to be biphasic, comprised of a rapid and a slow component. The slopes of the rapid components, 0.39 and 0.37 per day from the 1st to the 6th day, are lower than those previously recorded when isotopic uric acid was administered, 0.46 to 0.76 (35, 36). The major process contributing to this phase of the decline is assumed to be the turnover of the body pool of uric acid. The slower process, manifest after the 7th day, presumably represents the turnover of uric acid precursors which had been labeled by the administration of AIC-C^{13}. Tissue purines may be among these precursors.

Effect of AIC upon Glycine Utilization—Included in the plans of the present experiments was the feeding of glycine-N^{15} together with AIC-C^{13}.

It is intended, after the collection of data from normal subjects, to secure comparable data from gouty subjects in order further to explore the "shunt" in uric acid synthesis which has been postulated in this disease (13). An unexpected finding was that the enrichment of uric acid with respect to N^{15} fell far below the values anticipated on the basis of earlier studies with normal subjects. That this finding was not due to metabolic peculiarities in the present subjects was demonstrated by repeat experiments (Part II) in which glycine-N^{15} without AIC was fed (Fig. 2). Under these circumstances, the plots of N^{15} enrichment in uric acid *versus* time were comparable with those previously presented (13). Comparison of

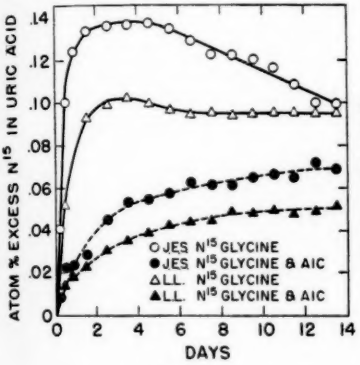

FIG. 2

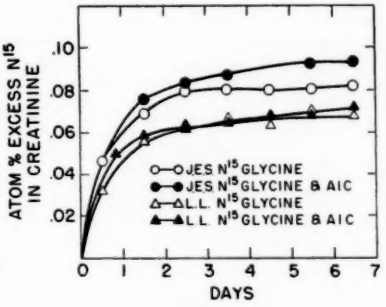

FIG. 3

FIG. 2. The concentration of N^{15} as atom per cent excess in urinary uric acid from two normal subjects receiving glycine-N^{15} with and without AIC.

FIG. 3. The concentration of N^{15} as atom per cent excess in urinary creatinine from two normal subjects receiving glycine-N^{15} with and without AIC.

the curves in Fig. 2 reveals that when glycine-N^{15} was fed together with AIC the uric acid was much poorer in N^{15} and the maximal N^{15} enrichment was achieved far later than when glycine alone was fed. Likewise, the recoveries of N^{15} in urinary uric acid were strikingly depressed by the feeding of AIC (see Table II).

This interference with the incorporation of dietary glycine nitrogen into urinary uric acid induced by the simultaneous feeding of about 1 gm. of AIC has been further explored. That AIC did not diminish the general metabolic availability of ingested glycine was indicated by the absence of an analogous depression of the labeling with N^{15} of urinary creatinine in the same studies (Fig. 3). The possibility that uric acid-N^{15} was diluted by products derived from AIC has been considered. Such a dilution should not affect the relative isotope abundances in positions 1, 3, 7, and 9 of the

uric acid excreted. Degradation studies, permitting the separation of nitrogen at positions 1 and 3 from that at positions 7 and 9, revealed a disproportionate depression of N^{15} concentration in positions 7 and 9 of uric acid consequent to AIC ingestion (Table III). Since glycine is known to contribute nitrogen specifically to position 7 of uric acid (37) and only secondarily to the remaining nitrogen positions, it would appear that AIC, fed in the present dosages, has specifically interfered with the incorporation of glycine into the purine nucleus of uric acid. Possibly related to this observation is the finding of Abrams (38) that adenine depresses

TABLE III

Uric Acid Degradation

Uric acid isolated from urine of the 2nd day in each study was degraded to separate the nitrogen in the 1,3 and 7,9 positions. The N^{15} concentration of each degradation product is given in Columns A and B. In Column D the N^{15} concentration determined on the intact uric acid is reported.

Study	Atom per cent excess N^{15}				Ratio $\frac{7,9}{1,3}$ $\frac{B}{A}$
	1,3 positions (A)	7,9 positions (B)	Calculated average, $\frac{A+B}{2}$ (C)	Analyzed uric acid (D)	
Part II. N^{15}-Glycine					
J. E. S.	0.037	0.197	0.117	0.134	5.3
L. L.	0.029	0.134	0.082	0.093	4.6
Part I. N^{15}-Glycine + AIC					
J. E. S	0.025	0.049	0.037	0.032	2.0
L. L.	0.018	0.038	0.028	0.022	2.1
Synthetic uric acid-1,3-N^{15}	58.7	0.014	29.4	28.3	2×10^{-4}

the incorporation of labeled glycine into nucleic acids of rat intestine and liver *in vivo*. Other evidence of a pharmacological action of AIC is its potentiation of the toxicity of 8-azaguanine and its inhibition of guanase (39).

SUMMARY

4-Amino-5-imidazolecarboxamide-4-C^{13} has been synthesized and fed to two normal adult males at a dosage level of 14 mg. per kilo. Approximately 20 per cent of the AIC fed was recovered unchanged, with its isotope undiluted, from the urine excreted in the first 8 hours. Between 20 and 23 per cent of the C^{13} was recovered in uric acid excreted in the 2 to 3 weeks following ingestion of AIC-C^{13}. The C^{13} concentration in uric acid was maximal at about 14 hours and declined rapidly thereafter. The

value of the maximum and the slope of the initial decline in C^{13} concentration indicate the existence of a mechanism in man for the prompt conversion of ingested AIC to uric acid. The data collected in later time periods suggest the existence of a second, less direct, mechanism whereby carbon from AIC enters uric acid, possibly via body purines.

The simultaneous administration of AIC and glycine-N^{15} was followed by the excretion in urinary uric acid of less N^{15} than appeared after glycine-N^{15} alone was given. Evidence is presented to indicate that AIC administration interferes with the synthesis of uric acid from glycine.

The authors express their indebtedness to Dr. J. Freund, who conducted histological studies on a rat fed AIC, to Miss Jean D. Benedict for cooperation in the synthesis of glycine-N^{15}, to Dr. James B. Wyngaarden for a sample of synthetic uric acid-1,3-N^{15}, to Mr. Frank J. Rennie, Miss Vida Klybas, Miss Dolores Lopez, and Mr. Arthur Kenney for performance of isotope analyses. Dr. Julius White and Mr. William Comstock of the National Cancer Institute generously permitted us to use their mass spectrometer for some of the N^{15} analyses.

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THE SULFHYDRYL GROUPS OF CRYSTALLINE PROTEINS

I. SOME ALBUMINS, ENZYMES, AND HEMOGLOBINS

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The amperometric titration of sulfhydryl groups in compounds of biological interest with silver in ammoniacal solution (1–5) has given some useful results. Its application to protein —SH groups, however, has been of limited value, because of three major drawbacks: (1) The high pH of the NH_4OH-NH_4NO_3 buffer used tends to denature proteins and accelerate the rate of oxidation of —SH groups. (2) The presence of ammonium ions is undesirable *per se*, as was pointed out previously (1), since they can cause the total disappearance of some protein —SH groups (6, 7). (3) Alcohol greatly improves the sharpness of the end-point and this has made it necessary to use this denaturing solvent in the titration of most proteins. All these factors combine to make the results obtained by this method difficult to interpret and are likely to lead to artifacts.

It has now been found that these objections can be overcome by the use of Tris[1] instead of ammonia as the agent to form the silver complex. This makes it possible to titrate protein —SH groups in *neutral, aqueous, buffered* solutions, with a buffer which, unlike the ammonia buffer, has not been shown to have any drastic effects on proteins. It will be shown that in this system the —SH groups of many proteins can be studied in the native state and that their relative accessibility and the effect of denaturing agents thereon are brought out very clearly by this procedure.

EXPERIMENTAL

Stability of Silver-Tris Complex and Its Reduction at Rotating Platinum Electrode

Ammonia has been used as a complex-forming agent in the titration of —SH groups in order to prevent the precipitation of silver by anions such as chloride. Moreover, the specificity of the method was thereby enhanced by preventing the reaction of silver with possible interfering substances such as purines and pyrimidines (2). An investigation of the stability of the silver-Tris complex has shown that it is of the same order

[1] In this paper tris(hydroxymethyl)aminomethane will be referred to as Tris.

of magnitude as that of the silver-ammonia complex and a hypothesis has been proposed to account for the relatively high reactivity of Tris towards silver (8). From this point of view Tris appears to be just as suitable as ammonia for the titration of —SH groups.

One consequence of the somewhat higher dissociation of the silver-Tris complex is the fact that its reduction at the rotating platinum electrode occurs at more positive potentials than that of the silver-ammonia complex. This difference in potential is made even larger by the fact that the concentration of Tris in the RNH_2 form used in the present method is only 0.02 M as compared with 0.25 M ammonia used previously. As can be seen from

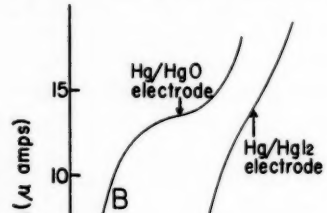

FIG. 1. Current-voltage curves of silver at the rotating platinum electrode. Curve A, silver wave in ammonia; Curve B, silver wave in Tris.

Fig. 1, this has the considerable advantage of extending the diffusion plateau over a much wider range of potentials (Curve B) and consequently much more satisfactory titrations are obtained. It should be emphasized that the curves in Fig. 1 were obtained in completely aqueous solutions and in the presence of oxygen and that under these conditions the diffusion plateau of the silver-ammonia complex (Curve A) is very ill defined.

Reference Electrode

It is convenient to carry out these amperometric titrations without the application of an external potential. This can be done by choosing a reference electrode which permits the reduction of the silver complex at zero-applied potential, so that the potential of the reference electrode coincides as nearly as possible with the middle of the diffusion plateau. In the case of the ammoniacal silver titration the Hg-HgI_2 electrode, with a potential of −0.23 volt *versus* the saturated calomel electrode, was chosen

for this purpose by Kolthoff and Harris (9). As can be seen from Fig. 1, a more positive electrode is, of course, required for amperometric silver titrations in Tris. It was found that the Hg-HgO-saturated $Ba(OH)_2$ electrode of Samuelson and Brown (10), which has a potential of −0.10 volt *versus* the saturated calomel electrode, fulfilled this purpose admirably. It is prepared by shaking an excess of solid red mercuric oxide and solid barium hydroxide with water for a few minutes. The electrode is made up in a vessel of the type described by Kolthoff and Harris (9). A layer of mercury is placed in the bottom of the flask, this is covered with a slurry of the solid HgO and $Ba(OH)_2$, and the vessel is filled up with the saturated supernatant solution. This electrode, when protected from light, was found to be stable for several months.

Apparatus and Procedure

The titrations were carried out in 100 ml. beakers containing a total volume of 30 ml. In this solution were immersed the rotating platinum electrode and the bridge from the reference electrode. This consisted of a short piece of glass tubing fitted with a rolled filter paper plug (Whatman No. 1) soaked in saturated KCl. The glass tubing was attached to a 60 cm. long piece of Tygon tubing which led to a No. 2 3-way stop-cock which, in turn, was connected to the reference electrode by a piece of Tygon tubing. The bridge was filled with the filtered electrode solution as far as the 3-way stop-cock and with saturated KCl from the stop-cock to the filter paper plug. By means of a funnel mounted on the stop-cock, the part of the bridge distal to the reference electrode could be washed out with saturated KCl and fitted with a new filter paper plug every day. The platinum electrode was rotated by a Sargent synchronous rotator. The galvanometer sensitivity was of the order of 0.1 to 0.2 μa. per mm. The titrating solution (1 or 2 × 10^{-3} M $AgNO_3$) was added from a syringe micro burette (Micro-Metric Instrument Company, Cleveland, Ohio).

Most of the work reported below was carried out in solutions which were 0.02 M in Tris in the RNH_2 form, 0.113 M in Tris in the RNH_3^+ form, and 0.01 M in KCl.[2] This titration mixture, of which the final pH was 7.4 at 25°, was prepared by mixing 4.0 ml. of 1.0 M Tris with 3.4 ml. of 1.0 M HNO_3 and 0.3 ml. of 1.0 M KCl. After the addition of a suitable concentration of the thiol (about 1 μmole), the solution was made up to 30 ml. and titrated. For the titration of non-protein —SH groups the presence of 0.01 per cent gelatin was found to give more reproducible current readings, since it promotes the deposition of silver in a finely divided form (11). This was unnecessary for the titration of proteins.

[2] KCl was added, since it was found to accelerate the rate of return of the current to zero, or nearly zero, at the beginning of the titration.

Source, Preparation, and Standardization of Materials

All chemicals, unless otherwise stated, were analytical reagent grade.

The Tris was Sigma "121," obtained from the Sigma Chemical Company, St. Louis, Missouri. $AgNO_3$ was Merck primary standard. 0.1 M solutions were made up by direct weighing and were diluted daily to give the desired concentration. Urea was Mallinckrodt analytical reagent which was further purified as described previously (12), *i.e.* by treating a concentrated solution at 50° with Amberlite MB-1 cation and anion exchange resin and allowing it to crystallize from the warm filtrate. This purification, as well as the use of deionized water for the preparation of all solutions, was found to be *absolutely essential*. Knox's unflavored No. 1 gelatin gave the most satisfactory results of several brands tried. *p*-Chloromercuribenzoic acid was purchased from the Sigma Chemical Company.

Glutathione was purchased from the Schwarz Laboratories, Inc., Mt. Vernon, New York, and *tert*-dodecyl mercaptan from Phillips Petroleum Company, Bartlesville, Oklahoma (manufacturer's assay = 98 per cent —SH).

Crystallized bovine plasma albumin was obtained from the Armour Laboratories, Chicago, Illinois, and human serum albumin, Cohn Fraction V, was donated by the American Red Cross.

Crystalline ovalbumin was prepared by the method of Kekwick and Cannan (13). The concentration of ovalbumin in the dialyzed solutions was determined by Kjeldahl nitrogen.

Canine and human oxyhemoglobin were crystallized as described by Drabkin (14). Excellent yields of highly crystalline hemoglobin were obtained by this method. Sheep hemoglobin was crystallized by a modification of the procedure of Marshall and Welker (15). The crystalline hemoglobin preparations were stored in their mother liquor. Before use, aliquots were removed by centrifugation and immediately dissolved in water. The resulting hemoglobin solutions were standardized both on the basis of their iron content (16) and their extinction at 540 and 576 mμ (17).

Creatine transphosphorylase (18) was a twice recrystallized, lyophilized sample kindly donated by Dr. L. Noda. Its activity was 51 units per mg. and the $\epsilon_{280}/\epsilon_{260}$ ratio was 1.87. The protein concentration was determined from the absorbancy at 280 mμ ($E^{1\%}_{1\,cm.} = 8.4$ (18)).

Crystalline D-glyceraldehyde-3-phosphate dehydrogenase was prepared from rabbit muscle as described by Cori, Slein, and Cori (19). Various preparations were recrystallized three to five times in the presence of 10^{-4} M Versene and 10^{-4} M cysteine ethyl ester at each stage. The last crop was dissolved in 10^{-4} M Tris buffer, pH 7.4, and was dialyzed against three changes of 10^{-4} M Versene and 10^{-4} M Tris buffer. This was followed by

dialysis for 10 hours against the Tris buffer alone. The protein concentration of the solutions was calculated from the absorbancy at 280 mμ and the extinction coefficient of $E^{1\%}_{1\,cm.} = 10.2$.[3]

Rabbit muscle aldolase was prepared by the method of Taylor, Green, and Cori (20). Various preparations were recrystallized two to four times from the required ammonium sulfate concentration with the addition of Versene and cysteine ethyl ester as described above. The protein concentration was calculated from the absorbancy at 280 mμ and the extinction coefficient of $E^{1\%}_{1\,cm.} = 9.10$.[3]

α-Chymotrypsin (21) and chymotrypsin B (22), each recrystallized three times, were kindly donated by Dr. M. Laskowski.

TABLE I

Titration of Simple Thiols

The conditions employed were 0.133 M Tris, pH 7.4, and 0.01 per cent gelatin.

Compound	No. of determinations	—SH	Maximal error
		per cent	*per cent*
tert-Dodecyl mercaptan	4	97.3	±1.0
Glutathione	12	97.7	±1.5
" in 8 M urea	2	98.0	0

Results

Specificity and Accuracy of Method

In order to test the specificity of the method, the following compounds were titrated with silver under the conditions described above in concentrations 10 times greater than those used for the titration of thiols: histidine, leucine, arginine, phenylalanine, serine, isoleucine, lysine, threonine, proline, tryptophan, aspartic acid, valine, cystine, alanine, glutamic acid, glycine, tyrosine, methionine, diphosphopyridine nucleotide, adenosine triphosphate, hemin chloride, ascorbic acid, and oxidized glutathione. All these substances gave a zero titration. In addition, 14 mg. samples of ribonucleic acid and deoxyribonucleic acid also gave a zero titration. Furthermore, 30 mg. of α-chymotrypsin and 22 mg. of chymotrypsin B were found to give titrations of less than 0.02 μeq.

The procedure was checked with *tert*-dodecyl mercaptan and glutathione under the conditions described above with the results shown in Table I. In addition, glutathione was titrated over the range pH 7.17 to 7.75 and with a total Tris concentration from 0.1 to 0.25 M with identical results. The concentration of the RNH_2 form of Tris was *always at least 0.02 M* in order to provide enough complex-forming agent for the silver.

[3] We are indebted to Dr. Sidney Velick for these values.

Proteins

General

The application of this method to proteins showed that, in addition to the determination of the total number of —SH groups per mole of protein, this procedure also gave clear cut evidence of the *reactivity* of the —SH

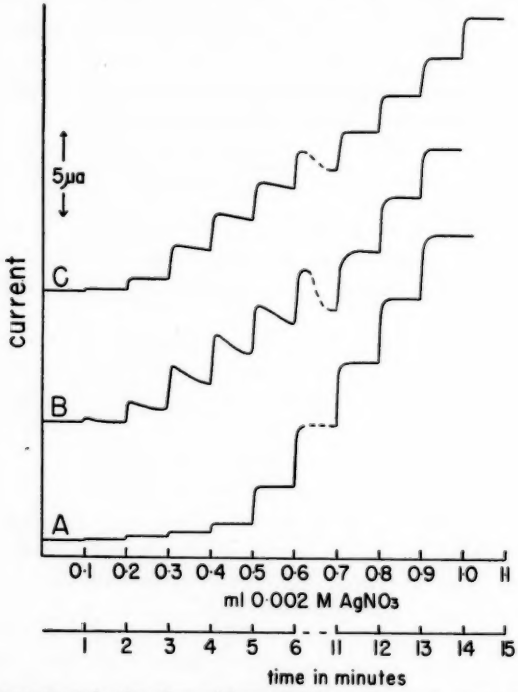

FIG. 2. Amperometric titration of hemoglobins. Curve A, sheep hemoglobin in Tris; Curve B, human hemoglobin in Tris; Curve C, human hemoglobin in Tris and 8 M urea.

groups under different conditions, in contrast to the ammoniacal silver titration, for which the drastic conditions employed precluded observations of this kind.

These differences are illustrated in Fig. 2, where silver concentration (in terms of current) is plotted against time, each point corresponding to the addition of an equal increment of silver nitrate. Curve A is an example of —SH groups which react so rapidly with silver that the current does not rise at all upon the addition of silver before the end-point, whereas beyond

the end-point each silver addition leads to an immediate rise in current which does not change with time. This is in marked contrast to the titration curves obtained with proteins in which the —SH groups are "masked" and therefore react only slowly with silver. In these cases, an extreme example of which is shown in Fig. 2, Curve B, the addition of silver even before the end-point produces an initial rise in current which gradually decreases towards an equilibrium value, showing that silver is being removed slowly by the —SH groups. As would be expected from mass action considerations, the rate of removal of silver increases with the silver concentration. After enough silver has been added and sufficient time has elapsed for complete reaction, further increments of silver nitrate produce a rise in current which no longer changes perceptibly with time. The difficulty of reaching complete reaction, as well as the longer extrapolation to the end-point, makes the results obtained with proteins containing such unreactive —SH groups less exact.

TABLE II

Sulfhydryl Content of Albumins and Hemoglobins

Protein	In Tris, pH 7.4		In Tris, pH 7.4, and urea, 8.0 M	
	No. of determinations	moles —SH per mole	No. of determinations	moles —SH per mole
Bovine serum albumin	8	0.67 ± 0.04	6	1.04 ± 0.04
Ovalbumin	5	5.0 ± 0.1	2	4.3
Sheep HbO_2	6	7.6 ± 0.3	1	7.8
Human "	7	9.5 ± 0.3	4	8.0 ± 0.2
Canine "	9	11.7 ± 0.4	2	10.3 ± 0.1

The proteins with unreactive —SH groups were therefore titrated in 8 M urea, having stood in the presence of this denaturing agent for about 30 minutes. This resulted in all cases in a dramatic increase in the reactivity of the —SH groups towards silver (compare Curve C with Curve B of Fig. 2). It should be emphasized that this high concentration of urea does not interfere with the titration *per se*, since identical results are obtained in water and in urea with glutathione (Table I) and with sheep hemoglobin, the —SH groups of which are fully reactive in water (Table II).

It will be seen from Table II that, with three of the proteins which had the least reactive —SH groups (ovalbumin, canine, and human hemoglobin), the result in water is actually 10 to 15 per cent higher than that in urea. This is probably due to the inaccuracy of the titration in water, the reasons for which have been discussed above.

In summary, the most accurate end-points with unreactive —SH groups are obtained by adding a slight excess of silver nitrate to the protein in 8 M urea, allowing about 30 minutes for complete reaction, and then completing the titration. The end-point is obtained by plotting current *versus* ml. of $AgNO_3$ and extrapolating to zero current.

Individual Proteins

The results obtained on bovine serum albumin and ovalbumin are in good agreement with previous findings (1, 23). Moreover, the —SH groups of bovine serum albumin were found to be very reactive and those of ovalbumin highly unreactive towards silver in water, which was also to be expected from earlier work. The increase in absolute —SH titer upon treatment of serum albumin with 8 M urea was, however, somewhat surprising, since Hughes' discovery of mercaptalbumin (24) has led to the assumption that serum albumin contains two-thirds of a protein containing one —SH group per mole, *i.e.* mercaptalbumin, and one-third of a non-SH-containing protein. The present results suggest, however, that the latter fraction also possesses one —SH group per mole, but of a far less reactive variety. In view of the unexpected nature of this result, the nitroprusside color of bovine serum albumin was compared in 8 M urea and in water and was indeed found to be much stronger in the former medium. In addition, excellent recoveries of glutathione from mixtures with bovine serum albumin in both media increase our confidence in the reality of these results. Similar increases in the —SH titer of human serum albumin were also observed in 8 M urea.

The hemoglobins showed a striking variation in the reactivity of the —SH groups among different species, since the —SH groups of sheep HbO_2 were found to be fully reactive, whereas those of dog, and particularly human HbO_2, were quite unreactive (*cf.* Fig. 2, Curves A and B). This reactivity did not change appreciably on conversion to the CO derivatives. In regard to the actual values of moles of —SH per mole of protein, those obtained in the presence of urea are considered the more reliable in the case of the unreactive hemoglobins of dog and man for the reasons discussed above. The figures of 8 moles of —SH per mole for sheep and human Hb and 10 moles of —SH per mole for canine Hb are, of course, much higher than any values previously reported, including those by Benesch and Benesch (25, 26). Amino acid analyses for cystine on hydrolysates of these proteins are of little value for purposes of comparison, since these figures are very uncertain, in view of the losses during hydrolysis (27). More confidence can be placed, however, in the total sulfur values reported for different species. Table III shows that, when the methionine values found by Brand and Grantham (28) are subtracted from

the total sulfur of two of the species investigated in the present study,[4] the difference amounts exactly to the moles of —SH per mole of protein reported here. This leads to the important conclusion that these *hemoglobins do not contain any disulfide bonds.* These results were further confirmed by an independent method, *i.e.* equilibrium dialysis against *p*-mercuribenzoate in phosphate buffer, pH 7.4.[5] The amount of *p*-mercuribenzoate bound after equilibration for 20 hours was determined by estimating the concentration of the mercurial in the outside solution from its absorbancy at 235 mμ (29). The results for human and sheep HbO_2 (Fig. 3) agreed with those of the amperometric method, since a plateau value of 8 moles per mole was reached in the plot of moles bound per mole of protein (r) against concentration (c). The further increase in binding beyond the plateau, *i.e.* when the protein is exposed to even higher concentrations of the mercurial, must be due to interaction of the mercurial with secondary binding sites other

TABLE III

Absence of Disulfide Bonds in Hemoglobin

Protein	Total S	Methionine	(Total S) − (methionine)	—SH found
	moles per mole	*moles per mole*	*moles per mole*	*moles per mole*
Human HbO_2..	14 (Belassa (33))	6 (Brand and Grantham (28))	8	8
Canine " ..	12 (Vickery (34))	2 (" " " (28))	10	10

than —SH. These observations illustrate the limited specificity of metallic —SH reagents when they are employed in excessive concentrations. A plot of $1/r$ against $1/c$ for the values up to and including the plateau revealed that the association constant for sheep hemoglobin was twice that for human hemoglobin (4.6×10^4 and 2.4×10^4, respectively). Thus, even in terms of free energy (as distinct from the kinetic differences in reactivity observed by the amperometric method), the —SH groups of sheep hemoglobin have a greater affinity for metals than those of human hemoglobin.

[4] The calculation could not be made for sheep hemoglobin, since Brand and Grantham give no methionine values for this protein.

[5] Since viscose sausage casing contains considerable amounts of sulfur in the form of xanthates and trithiocarbonates, which readily react with heavy metals, the dialyzing membranes were washed thoroughly, first in running water and then for about a week in frequent changes of distilled water. As a result, the amount of mercurial bound by the dialyzing bags became quite constant, amounting to 10 per cent of the free concentration present. Corrections for this binding were made accordingly.

XUM

Some reports on direct —SH determinations of hemoglobin require further comment. Hughes (24) reported two —SH groups per mole of human hemoglobin. It is probable that this represents only the two most reactive —SH groups and that the others escaped detection, owing to the slow equilibration commented on by the author himself. Ingbar and Kass (3) found 2 to 5 moles of —SH per mole of human hemoglobin by the ammoniacal silver titration. Two main factors would tend to produce low results by this procedure: (1) slow reaction with the silver and there-

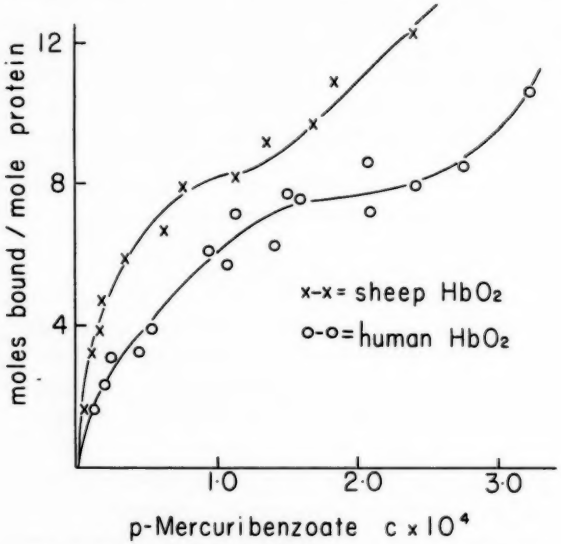

FIG. 3. Reaction of hemoglobins with *p*-mercuribenzoate

fore incomplete equilibration and (2) the specific effect of ammonium ions in causing —SH groups to "disappear" in some proteins, a fact first reported with myosin by Greenstein and Edsall (6, 7). In view of these results, the possibility of artifacts in the *ammoniacal* silver titration was already pointed out earlier (1). These factors are also responsible for an earlier report of four —SH groups per mole of dog hemoglobin (25). Titrations with the Tris method show clearly that the "ammonium effect" is operative with hemoglobins. This is best illustrated with sheep hemoglobin in which slow equilibration does not constitute a complicating factor. The data in Table IV demonstrate to what extent the presence of ammonia can affect the —SH titer.

Creatine transphosphorylase presents a particularly clear cut case from

the point of view of the reactivity of its —SH groups, since the first two —SH groups were found to be fully reactive, while the rest are fairly unreactive (Table V). This is reminiscent of the *a* and *b* groups of urease described by Hellerman *et al.* (30), but it remains to be tested how enzy-

TABLE IV

Effect of Ammonium Ions on —SH Groups of Sheep Hemoglobin

Conditions	moles —SH per mole
Tris 0.133 M, final pH 7.4	7.6
" 0.133 " NH_4NO_3 1.7 M, final pH 7.4	6.3
" 0.133 " pH 10.2	7.4
NH_4OH 0.25 M, NH_4NO_3 0.05 M, pH 10.1, NaCl 0.85%	5.0

TABLE V

Sulfhydryl Groups of Crystalline Enzymes

The values, expressed in equivalents, are averages of at least triplicate titrations in each case.

	Times crystallized			
	2	3	4	5
Creatine transphosphorylase (81,000 gm.)				
Ag^+ bound rapidly		2		
Total Ag^+ bound		6.5		
Aldolase (147,000 gm.)				
Ag^+ bound in Tris	20.6	20	23	
" " " " + 8 M urea	28.8	29	28.5	
D-Glyceraldehyde-3-phosphate dehydrogenase (120,000 gm.)				
Ag^+ bound	11	11	11	11

matic activity might be correlated with these two sets of —SH groups. It is noteworthy that the —SH titration of this enzyme is unaffected by ammonium salts.

D-*Glyceraldehyde-3-phosphate dehydrogenase* was found (Table V) to contain eleven —SH groups per mole, two of which were less reactive than the rest. The total number of —SH groups found (which remained the same in urea) is in good agreement with the value reported by Boyer and Segal (31) using *p*-mercuribenzoate. Three separate preparations of enzyme,

crystallized four times, gave values of 10.9, 11.0, and 10.9 equivalents of —SH per 120,000 gm. of protein.

Aldolase was found to behave much like serum albumin in that a stable end-point was reached in Tris when approximately 20 equivalents of Ag^+ had been bound, but denaturation with 8 M urea made available an additional 9 equivalents (Table V). The number of apparently readily available —SH groups varied somewhat, but the total number of —SH groups was remarkably consistent from preparation to preparation. Results nearly identical with those shown in Table V were obtained with a second preparation. The silver complex of aldolase was the only one found to precipitate in the titration mixture after the theoretical amount of silver had been added. This is consistent with the extremely high concentration of —SH groups found in this protein. The precipitate did not form in solutions containing 8 M urea.

Work is in progress on the relation of silver binding to catalytic activity of these proteins.

DISCUSSION

A consideration of some of the special features which combine to make the method used for this work especially suitable for the study of —SH groups in proteins seems appropriate.

The titrations are carried out under extremely mild conditions, *i.e.* in neutral, aqueous solution, in which denaturation of proteins is kept to a minimum. On the other hand, the effect of urea denaturation can be followed under controlled conditions without intrinsic effect on the accuracy of the method.

The procedure yields information not only on the concentration of —SH groups in proteins, but also on their relative reactivity.

The specificity of the method for —SH groups is of a high order for a number of reasons: Owing to the high sensitivity of the rotating platinum electrode, the absolute concentration of protein, and therefore of silver, is extremely low (about 2×10^{-5} M). This may be contrasted, for example, with equilibrium dialysis against *p*-mercuribenzoate, when concentrations 10 times higher had to be employed to obtain significant results. Moreover, only a comparatively small excess of silver is necessary for an accurate determination of the end-point. Finally, since the silver is used in the form of a relatively stable complex, it will have little tendency *per se* to react with groups other than —SH groups.

SUMMARY

1. A new amperometric method is described for the titration of —SH groups in neutral, aqueous, buffered solution, with $Ag(Tris)_2^+$ as the titrat-

ing agent. It is shown to be sensitive, accurate, and highly specific for —SH groups. It is especially useful for the study of proteins, since both the number and the reactivity of the —SH groups can be determined in native and denatured proteins.

2. Crystallized bovine serum albumin contains two-thirds of an —SH group per mole, but upon urea denaturation one —SH group per mole is found. This suggests the presence of one —SH group per mole in both fractions of serum albumin, an easily accessible one in mercaptalbumin, and a much less reactive one in the remaining fraction.

3. Titrations of crystalline hemoglobins show the presence of 8 moles of —SH per mole in sheep and human hemoglobin and 10 moles of —SH per mole in canine hemoglobin. The —SH groups of sheep hemoglobin are fully reactive, while a major proportion of those of canine and human hemoglobin is highly inaccessible. It is concluded that the hemoglobins of dog and man do not contain any disulfide bonds.

4. Creatine transphosphorylase contains two reactive and four unreactive —SH groups. D-Glyceraldehyde-3-phosphate dehydrogenase contains eleven —SH groups per mole, two of which are somewhat less reactive than the rest. Aldolase contains approximately twenty reactive —SH groups and an additional number, making a total of twenty-nine, become available only upon denaturation with urea.

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Addendum—Since the submission of this paper, Ingram (32) has reported a comprehensive study of the —SH groups of hemoglobins from several different species. There is a remarkable agreement between his results and those reported here, in spite of the different methods used.

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STEROID BIOSYNTHESIS BY SURVIVING TESTICULAR TUMOR TISSUE*

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The appearance of gynecomastia and other feminizing symptoms in the male concomitant with the appearance of certain types of testicular tumors has long been noted (1). Therefore, the possibility has been suggested that these tumors produce excessive amounts of female sex hormones. The recent work of Goldzieher and Roberts (2) demonstrated that human testes contain small amounts (5.7 γ per kilo of tissue) of estradiol-17β. More recently, Marti and Heusser (3) obtained a large testicular tumor from which they were able to isolate 13 mg. of pure estradiol as the dibenzoate. The tissue of this tumor was identified by pathologic examination as having been displaced adrenal tissue.

In 1945, Laipply and Shipley (4) studied a case of extragenital choriocarcinoma and reported isolation of pregnanediol and estrogens from the urine of this patient in an amount which greatly exceeded that of normal urine. Guinet *et al.* (5) also reported an elevation of pregnanediol in the urine of a patient with a testicular tumor.

From the Department of Pathology at Massachusetts Memorial Hospitals a piece of tumor tissue weighing 1.4 gm. was obtained which was described as an embryonal testicular carcinoma with lymphatic invasion. After incubation of the tumor tissue with 1-C^{14}-acetate, the mixture yielded radioactive testosterone, androstenedione, progesterone, estrone, and estradiol. Urine specimens, taken postoperatively from this patient, yielded excessive amounts of pregnanediol.

In order to rule out the possibility of the presence of small but highly radioactive impurities, the radioactive steroid fractions were subjected to repeated chromatography on paper, treatment with chromic acid followed by crystallization of the oxidation product, formation, chromatographic purification, and crystallization of a derivative, and finally reformation and crystallization of the free steroids.

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It is felt that these manipulations provide reasonable assurance that the radioactivity is associated with the crystalline steroids and not with some chemical impurity.

Methods

Incubation—The tissue (0.8 gm.) was sliced in a Stadie-Riggs microtome and added to a flask containing 100 ml. of Krebs-Ringer-phosphate buffer at pH 7.3, 2 μc. of 1-C^{14}-acetate, 10,000 units of Antuitrin, and 20,000 units of penicillin G. The mixture was aerobically incubated for 17 hours in a water bath at 38° with shaking.

Extraction—The contents of the flask were homogenized in a Waring blendor and centrifuged, after which the supernatant fluid was decanted. Each of the two fractions was extracted four times with chloroform, and the extracts were combined. After removal of the solvent *in vacuo*, 5 mg. each of testosterone, androstenedione, progesterone, estrone, and estradiol were added as carrier. In the accompanying diagram the major steps in the separation, purification, and characterization of the various steroid fractions are demonstrated schematically.

Measurements of Radioactivity—All radioactivity measurements were made by preparing planchets containing from 2 to 10 per cent of the total extracts. Counts were determined with a Tracerlab SC-16 windowless flow counter for a period of time to assure accuracy of ± 5 per cent. The values are reported as corrected counts.

Partitioning—The mixture was partitioned by an eight transfer counter-current distribution between pentane and 90 per cent methanol; the combined contents (100 ml.) of each funnel were then evaporated to dryness *in vacuo*, and the residues were checked for radioactivity in a flow counter. The distribution of activity of the various fractions is shown in Table I.

The combined fractions from funnels 4 to 8 were again partitioned by an eight transfer counter-current distribution between 1 N sodium hydroxide and toluene. The aqueous layers were brought to pH 8, and each was extracted four times with 15 ml. of toluene. The toluene extracts from the respective funnels were combined and evaporated to dryness *in vacuo*. Their radioactivity was then measured in a flow counter. Table II shows the distribution of activity and weights of the nine fractions after the partitioning.

Neutral Fraction

Chromatographic Separation—The extracts from funnels 0 to 2 were combined, dissolved in a few ml. of methanol, and applied to a paper strip 15 × 52 cm., which had previously been dipped in a 50 per cent methanolic propylene glycol mixture and then blotted lightly. The chromatogram

Tissue incubation extract
8 transfer partition
90% MeOH-pentane
Cholesterol, etc., 4200 c.p.m.
8 transfer partition, 10,625 c.p.m.
in NaOH-toluene
Phenolic fraction, 2650 c.p.m.
Neutral fraction, 6950 c.p.m.
Paper chromatographed, 3 times
Silica gel chromatogram
Testosterone, 472 c.p.m. per mg.
Androstenedione, 262 c.p.m. per mg.
Progesterone, 690 c.p.m. per mg.
CrO_3
CrO_3
Paper chromatography, twice
"Testosterone" (androstenedione)
Androstenedione
Progesterone
Estrone, 194 c.p.m. per mg.
Estradiol, 100 c.p.m. per mg.
"Testosterone" DNPH, 513 c.p.m. per mg.
Androstenedione DNPH, 500 c.p.m. per mg.
Progesterone DNPH, 404 c.p.m. per mg.
Estrone DNPH, 212 c.p.m. per mg.
Estradiol dibenzoate, 117 c.p.m. per mg.
"Testosterone," 506 c.p.m. per mg.
Androstenedione, 470 c.p.m. per mg.
Progesterone, 404 c.p.m. per mg.
Estrone, 210 c.p.m. per mg.
Estradiol, 115 c.p.m. per mg.

was developed in a ligroin-propylene glycol system (6) for 6 hours. With adequate precautions (7), the former was dried, after which 0.5 cm. paper strips were cut from each side of the chromatogram. These two strips

TABLE I

Distribution of Radioactivity after Partitioning between Pentane and 90 Per Cent Methanol

Funnel No.	Sample weight	Total counts	C.p.m. per mg.
	mg.		
0	43	2,125	49
1	18	625	34
2	12	875	73
3	11	625	57
4	21	2,125	101
5	12	1,875	156
6	18	1,500	83
7	26	2,375	91
8	79	2,750	35
Total	240	14,875	

TABLE II

Distribution of Radioactivity after Partitioning between Toluene and 1 N Sodium Hydroxide

Funnel No.	Sample weight	Total counts	C.p.m. per mg.
	mg.		
0	25	950	38
1	23	3,750	163
2	19	2,250	118
3	22	550	25
4	18	550	30
5	14	600	43
6	16	850	53
7	16	300	19
8	11	1,500	137
Total	164	11,300	

were then stained with Zimmermann's reagent. The area containing progesterone was cut from the paper chromatogram and eluted with methanol. The remainder of the paper was also eluted with methanol, reapplied to another 15 cm. paper strip, and then developed in a ligroin-propylene glycol system for 16 hours. After staining the end strips with

Zimmermann's reagent, the area containing androstenedione was eluted from the chromatogram. The remaining material was again eluted and rechromatographed for 32 hours as before, and the area containing testosterone was then eluted. Each of these fractions was again chromatographed in a ligroin-propylene glycol system for the amounts of time specified above, progesterone for 6 hours, androstenedione for 16 hours, and testosterone for 32 hours. After elution of the proper fractions they were once more subjected to paper chromatographic separation in the same system.

The final eluates of each of the three compounds were evaporated to dryness *in vacuo*. The residues were yellow and appeared as mixtures of crystals in some oil. The fractions were chromatographed on columns 5 mm. wide and packed with 500 mg. of silica gel (Davison 100 to 200 mesh). The steroid was applied in benzene and successively eluted with 3 ml. each of benzene, benzene-ethyl acetate, 19:1, 9:1, 4:1, and, finally, ethyl acetate. Eluates were collected in 1 ml. fractions.

From the testosterone chromatogram two fractions were obtained. The first was an oily residue in tubes 1 and 2, while tubes 8 to 12 contained a crystalline residue. For androstenedione a similar pattern was obtained, except that the crystalline material appeared in tubes 6 to 9. Progesterone was chromatographed on a column as described above; elution was carried out with ligroin, ligroin-benzene, 1:1, benzene, benzene-ethyl acetate, 19:1, 9:1, and, finally, ethyl acetate. Tubes 1 and 2 yielded an oily residue, tubes 6 to 9 yielded crystalline material, and tubes 17 and 18 yielded a brown oil.

The crystalline fractions were combined and counted in a flow counter. The radioactivity of the fractions was as follows: androstenedione, 5.9 mg., 1536 c.p.m., 262 c.p.m. per mg.; testosterone, 3.0 mg., 1408 c.p.m., 472 c.p.m. per mg.; progesterone, 3.0 mg., 2060 c.p.m., 690 c.p.m. per mg.

In order to rule out further the possibility of the presence of small but highly radioactive impurities, the crystalline fractions obtained were subjected to mild oxidation with chromic anhydride. This reaction does not significantly affect androstenedione and progesterone, but largely converts testosterone into androstenedione. Therefore, the physical data presented for this fraction henceforth pertain to androstenedione, although this represents the testosterone fraction. To call attention to this fact, this fraction will be referred to as "testosterone."

Because of the relatively high counts per minute and small weight of the progesterone fraction, this was further diluted with 3 mg. of authentic carrier.

Oxidation—Each of the three fractions was treated with 3 mg. of chromic anhydride in 10 ml. of glacial acetic acid at room temperature for 2 hours.

After this period 25 ml. of water were added, and the mixtures were left standing for 25 minutes. The steroids were then extracted with six 25 ml. portions of methylene chloride. The extracts were dried over anhydrous sodium sulfate and taken to dryness *in vacuo*. The "testosterone" fraction, weighing 4.3 mg. and having 565 c.p.m. per mg., and the androstenedione fraction both were crystalline. From the progesterone oxidation

TABLE III

Column Chromatography of Progesterone Fraction after CrO_3 Treatment

Solvent	Weight	C.p.m. per mg.	Residue
	mg.		
Ligroin	7.1	81	Oil
Ligroin-benzene, 1:1			
Benzene	2.7	200*	Crystals
Benzene-ethyl acetate, 9:1	4.6	52	Oil
" " 2:1			
Ethyl acetate	2.4	40	"
Methanol			

* Because of extra dilution with carrier, this represents 400 c.p.m. per mg.

TABLE IV

Silica Gel Column Chromatography of Phenolic Fraction

Fraction	Tubes	Weight	C.p.m.	C.p.m. per mg.
		mg.		
A	1– 5	3.7	307	83
B	6–14	1.3	134	103
C	15–20	2.4	36	15
D	21–27	2.5	293	117
E	28–32	1.4	105	75
F	33–49	2.3	69	30
G	50–55	5.6	0	0

an oily residue was obtained which was chromatographed on a column, as described before, of 500 ml. of silica gel. The sample was applied to the column with a minimum of ligroin and eluted with ligroin, benzene, ethyl acetate, and methanol, as shown in Table III.

Phenolic Fraction

The contents from funnels 6 to 8 from the partitioning between 1 N sodium hydroxide and toluene (Table II) were combined. The mixture, weighing 43 mg., was applied to a silica gel column (1 gm.), and 5 ml. frac-

tions were collected with an automatic fraction collector. The chromatogram was developed with 75 ml. of benzene, followed by benzene-ethyl acetate, 90 ml. 19:1, 35 ml. 9:1, 25 ml. 7:1, and 25 ml. 4:1, and was washed with 30 ml. of methanol. After evaporation of the solvents the results recorded in Table IV were obtained.

Fractions A and B and Fractions C, D, and E were combined. The former mixture, weighing 5.0 mg., was applied to a paper strip 6 cm. wide, and the latter, weighing 6.3 mg., to a 5 cm. paper strip which had previously been treated with a 50:50 mixture of methanol and propylene glycol. The chromatogram was developed in a toluene-propylene glycol system (8) for 16 hours, the run-off being collected in beakers placed under the strips. After drying in an oven at 60° for ½ hour, the edges (0.5 cm.) of the strips were trimmed and stained with ferric chloride-ferrocyanide reagent (9). Similarly, control chromatograms of estrone and estradiol mixtures were stained, and the areas corresponding to those two steroids on the original chromatogram, as well as starting line material, were cut from the strip and eluted with benzene. Each of these four fractions was then rechromatographed on paper in the same system and was stained as before. The run-off was developed for 4 hours, and the three other fractions were developed for 16 hours. The areas which stained were eluted from the chromatogram with benzene, washed twice with small amounts of water, and evaporated *in vacuo*, followed by drying in a stream of nitrogen. The samples were weighed and counted in a flow counter yielding the following results.

Sample	Weight, mg.	C.p.m.	C.p.m. per mg.
Starting line	4.5	63	14
Estradiol	2.1	231	100
Estrone	2.7	522	194

Preparation of Derivatives (10–12)

Experiments were performed to determine optimal conditions for the preparation of derivatives on amounts of material approximating that obtained from the incubation mixture. Dinitrophenylhydrazones (DNPH) were prepared from androstenedione, progesterone, and estrone. The dibenzoate of estradiol was also prepared. After chromatography on silica gel, followed by recrystallization, the uncorrected melting points were, respectively, 263–268°, 284–287°, 278–287°, and 169–170°. Ultraviolet absorption maxima were found to be, respectively, at 379.5, 381, and 440 mμ for estrone DNPH.

In a typical experiment 5 mg. of androstenedione were dissolved in 0.5

ml. of 95 per cent ethanol in a 15 ml. centrifuge tube. 0.5 ml. of a saturated alcoholic solution of 2,4-dinitrophenylhydrazine was added and the tube heated on the steam bath for 5 minutes. 4 drops of concentrated hydrochloric acid were added, and the mixture was heated 5 more minutes. To the solution water was added until a precipitate formed. The mixture was then extracted with chloroform after dilution with 25 ml. of water. The dried extract was evaporated to dryness *in vacuo*. The extract was chromatographed on 500 mg. of silica gel in a 5 mm. column.

TABLE V

Characterization of Derivatives

Melting points (uncorrected).

	Progesterone DNPH	Androstenedione DNPH	"Testosterone" DNPH	Estrone DNPH	Estradiol dibenzoate
M.p., °C.	280–283	263–266	261–264	274–279	168–170
Mixed with authentic derivative, °C.	283–287	264–267	264–267	278–280	169–170
Mixed with DNPH, °C.	218–231	210–219	209–223	214–231	
Ultraviolet maxima					
Observed, mμ	380.5	381	380.5	440	
Reported, mμ	380	380	380	440	
Infra-red spectra	Identity of products derived from incubation with authentic substances established				
Weight as free steroid, mg.	4.0	3.4	3.0	1.5	1.7
C.p.m. per mg. as free steroid	202*	500	513	212	117

* Because of the extra dilution with carrier, this is equivalent to 404 c.p.m. per mg. as compared with the activity of the other fractions.

The large orange-colored fraction eluted with ligroin-benzene, 7:1, was recrystallized three times.

Estradiol-17β (4 mg.) was treated with 1 ml. of pyridine and 0.5 ml. of benzoyl chloride at 0° for 3 hours, followed by standing at room temperature for 48 hours. The mixture was extracted with ether after pouring over ice. After washings with hydrochloric acid and sodium bicarbonate, the extract was evaporated to dryness. The residue was chromatographed on 200 mg. of silica gel, yielding a semicrystalline material in the fraction eluted with ligroin-benzene, 14:1. The material was recrystallized twice from ethanol.

The steroid fractions isolated from the incubation were treated according to the respective procedures described above. The melting points, specific activities, and spectral data are recorded in Table V.

Hydrolysis of Derivatives

The previously prepared progesterone DNPH (4 mg.) was treated with 10 ml. of ethanolic hydrochloric acid (3:1) and refluxed. After the material was completely in solution, 2.5 ml. of water were added, and refluxing was continued 4 hours. After cooling, 25 ml. of water were added, and the mixture was extracted with ether. After washing with acid and water, the dried extract was evaporated to dryness *in vacuo*. Chromatography on silica gel (500 mg.) yielded white crystals in the benzene-ethyl acetate, 14:1, eluate, shown to be progesterone by mixed melting point and infra-red spectra.

TABLE VI

Characterization of Hydrolysates from Derivatives

Melting points (uncorrected).

	Progesterone	Androstenedione	"Testosterone"	Estrone	Estradiol
M.p. of hydrolysate, °C.	120–122	168–170	170–172	260–262	175–177
Authentic steroid, °C.	121.5–122	171–171.5	171–171.5	259–260	178
Mixed with authentic steroids, °C.	121–122	171–172	171–172	260–261	176–177
Weight, *mg.*	1.1	0.95	1.05	0.95	0.74
C.p.m. per mg.	202*	470	506	210	115
Infra-red spectra	Identity of products derived from incubation with authentic substances established				

* Because of the extra dilution with carrier this is equivalent to 404 c.p.m. per mg. as compared with the activity of the other fractions.

Authentic estradiol-17β-dibenzoate (4 mg.) was refluxed with pyridine (1 ml.) for 30 minutes. After cooling and addition of 15 ml. of water and 5 ml. of benzene, the mixture was left standing for 24 hours. After removal of the benzene and further extraction with benzene, the organic layer was washed with acid, water, and sodium bicarbonate, followed by evaporation to dryness. The residue was chromatographed on 100 mg. of silica gel, and the fraction was eluted with benzene-ethyl acetate containing the free steroid as proved by mixed melting points and infra-red spectra.

One-half of each of the derivatives prepared from the incubation mixture was treated by the respective procedure outlined above. Their melting points, mixed melting points, spectral data, and specific activities are recorded in Table VI.

Isolation of Pregnanediol-3α,20α from Urine

An assay for pregnanediol was carried out on 1 liter of urine from a 48 hour collection obtained 6 months postoperatively from the patient whose

tumor was utilized for this study. Hydrolysis, extraction, and isolation of pregnanediol were performed according to the method of Astwood and Jones (13). White crystals, weighing 0.85 mg. and melting at 234–237°, were obtained. An infra-red absorption spectrum of this substance was identical with that of an authentic sample (courtesy of Dr. K. Savard) of pregnanediol-3α,20α.

A second assay on a urine specimen obtained 8 months after operation yielded 0.68 mg. of pure pregnanediol.

DISCUSSION

The results of Laipply and Shipley (4) and of Guinet *et al.* (5) have already indicated the probability that certain human testicular tumors produce progesterone, as is evidenced by the urinary excretion of pregnanediol.

The isolation of radioactive progesterone, estradiol, and estrone in the experiments reported here verifies the preceding assumption, since the surviving tissues produced these steroids and postoperative urinary studies on the patient led to the isolation of unusually large amounts of pregnanediol.

The radioactive counts for the five steroid fractions remained within the limits of error (±5 per cent) from the paper chromatographic separation through oxidation, derivative formation, and hydrolysis of the derivative, with two exceptions. The counts for the androstenedione fraction (262 c.p.m. per mg.) immediately after paper chromatography were low because of the presence of impurities. As can be seen, the total fraction weighed about 1 mg. more than even the total carrier added. The total counts of this fraction (1536 c.p.m.) are in line with the counts for testosterone (1408 c.p.m.). The drop from 690 to 404 c.p.m. per mg. for progesterone can be readily explained as being due to a radioactive impurity which was removed through the treatment with chromic acid.

From the activity of the final fractions it appears that the tissues were able to synthesize androstenedione, testosterone, and progesterone from acetate at approximately the same rate. The activity of the estrogenic fractions is about one-fourth as great.

While the nature of the tissue involved in this experiment apparently differs in its pathology from that reported by Laipply and Shipley and by Guinet *et al.*, these findings seem to reflect a possible over-all unanimity with respect to steroidogenesis, particularly the biosynthesis of progesterone.

From the practical point of view it appears possible that the progress of the disease may be traced through successive urinary assays for pregnanediol. This is further substantiated by the fact that the patient who do-

nated the tissue for this experiment is still excreting large amounts of pregnanediol after removal of the primary tumor. The pregnanediol is apparently produced by proved metastases.

SUMMARY

1. Incubation of surviving tissues from an embryonal tumor of the testes with 1-C^{14}-acetate resulted in the isolation of radioactive testosterone, androstenedione, progesterone, estradiol, and estrone.

2. Pregnanediol assays on postoperative urine specimens in the presence of known metastatic disease yielded higher than normal amounts of this steroid.

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STUDIES ON NATURALLY OCCURRING α-GLYCEROL ETHERS*

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The occurrence of the true ether linkage in nature is, in contrast to that of the acetal, amide, and ester links, very limited. The most prominent types of biological compound that are true ethers are (*a*) members of the group containing guiacol, vanillin, eugenol, and other methyl phenyl ethers; (*b*) thyroxine and allied compounds which are diphenyl ethers; (*c*) ethers of fatty alcohols and glycerol such as batyl and selachyl alcohols, which are α-monoethers of glycerol and have the general formula $R—O—CH_2—CH(OH)CH_2OH$.

Considerable attention has been devoted to the fatty ethers of glycerol with respect to their distribution in nature (1, 2–6) and their chemical constitution (1, 7–9). Some work has been carried out on their pharmacological nature; for example, their central depressant action (10), erythropoietic effect (11), and tuberculostatic activity (12). Little, however, is known of their metabolic relations. This is somewhat surprising in view of the fact that fatty acid esters of these compounds constitute major components of the liver and muscle lipides of several groups of lower elasmobranchs and are found to some extent throughout the teleosts and other phyla, including mammals (1, 3).

In the present work an attempt was made to establish a convenient method for the determination of α-glycerol ethers and to study the pattern of incorporation of C^{14} into glycerol ethers from usual lipide precursors, such as glycerol and acetate. In particular, the incorporation into glycerol ethers of C^{14} from these precursors has been compared with that into fatty acids.

EXPERIMENTAL

Biological Material—All marine material was obtained alive or, in the case of some sharks very shortly after death, at the Woods Hole Marine Biological Laboratory. Starfish were also shipped alive in sea water and utilized in Boston.

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Extraction of Fats—Fats were extracted by the methods of Bloor (13) and of Folch *et al.* (14) and by extraction with chloroform in a Soxhlet apparatus after thorough grinding with a 3-fold amount of $MgSO_4$ by weight. In the case of some elasmobranchs examined, results were obtained by all three methods and were found to be closely comparable.

Isolation and Determination of Unsaponifiable Fraction—The method of the Society of Public Analysts (15) was used. When only small amounts of fat were available, quantities were suitably adjusted throughout.

Determination of α-Glycerol Ethers—Glycerol ethers were determined by selective oxidation of the unsaponifiable fraction in a suitable medium with periodic acid, destruction of excess reagent, removal of the fatty material by precipitation with water and filtration, and estimation of formaldehyde generated by the standard colorimetric techniques of MacFadyen (16) and Tannenbaum and Bricker (17). The former method has been found more suitable and has been in routine use in this laboratory. Details of the determination follow.

Reagents—Periodic acid (250 mg. per 25 ml. of aldehyde-free 95 per cent EtOH, freshly prepared daily).

HCl, 1 N.

Sodium-arsenite, 1.2 N.

Celite.

Chromotropic acid reagent according to MacFadyen (16).

The unsaponifiable fraction isolated as above was dissolved in alcohol (95 per cent) or 50 per cent ethanol-50 per cent ethyl acetate. The optimal concentration of glycerol ethers, as batyl alcohol, was 1.74 mg. per ml. (5 μmoles per ml.).

1 ml. of the solution under test was pipetted into a tube graduated at 10 ml., and 1 ml. of periodic acid solution was added. The solutions were mixed with a thin, mushroom-tipped glass rod, which was left in the tube. After 1 hour the oxidation was terminated by adding in succession 3 ml. of distilled water, 1.5 ml. of N HCl, and 0.5 ml. of 1.2 N sodium arsenite. The solution was thoroughly mixed and allowed to stand until the yellow color had discharged. The stirring rod was then rinsed with distilled water and removed from the tube. The total volume was now made to 10 ml. with distilled water, and 0.5 gm. of Celite was added. The mixture was stirred thoroughly with a clean, dry, mushroom-ended glass rod and poured through a small fluted filter (Whatman No. 42) into a clean, dry tube.

0.5 ml. aliquots were then removed for formaldehyde determination by the MacFadyen method (16). In this laboratory, readings were made in a Klett colorimeter with a No. 56 filter. Color development was carried out exactly as recommended by MacFadyen. Blanks were performed on the complete system without the unsaponifiable fraction. The concentra-

tion of iodide was sufficiently low to obviate any interference from this source.

Standards—When pure batyl alcohol is available, it is undoubtedly the most suitable standard. Mannitol, glycerol, or formaldehyde, which are readily available, may also be used as standards, as follows: 1 ml. of a solution of the substance in distilled water (2.5 μmoles per ml. for mannitol or glycerol; 5 μmoles per ml. for formaldehyde) was added to 1 ml. of the solvent used for the lipide. 1 ml. of periodic acid (44 μmoles) was then added and the procedure carried out exactly as above. Mannitol, which may be obtained in a high degree of purity, is preferable to glycerol or formaldehyde since it constitutes a primary standard, whereas glycerol and formaldehyde solutions must themselves be standardized by an independent method.[1]

Preparation of Specimens of Purified Saturated Glycerol Ethers—Samples were prepared from two sources: (*a*) the unsaponifiable fraction of starfish (*Asterias forbesi*) diverticulum fat; (*b*) the unsaponifiable fraction of tiger shark (*Galeocerdo cuvier*) liver fat.

Starfish unsaponifiable fractions were treated by the method of Schlenk and Holman (19) to form a crystalline urea inclusion product of long chain aliphatic compounds. This was removed by filtration and twice recrystallized from isopropyl alcohol. It was then decomposed with water, the lipide extracted with ether, and twice recrystallized from ethyl acetate. The process was carried out on two different batches of starfish diverticulum fat, and the products melted at 68° and 70°, respectively. Further recrystallization did not raise the melting point, and some chimyl or other solid glycerol ether of chain length less than batyl alcohol was probably present. The urea inclusion product contained approximately 16 moles of urea per mole of glycerol ether (19).

A crystalline α-glycerol ether was prepared from tiger shark liver fat by methods similar to those previously described (2, 9), involving separation of steroids from the non-saponifiable fraction, hydrogenation of the steroid-free residue, and frequent recrystallization of the product. The product

[1] The method described has also been used for the determination of glycerol and 1,2-propanediol in experiments on blood preservation, of glycerol in metabolic experiments *in vitro*, and of mannitol in determinations of body fluid compartments. In these cases, aqueous solutions were used throughout, and the periodate oxidation was carried out in the presence of 1 ml. of N $NaHCO_3$. The addition of Celite and filtration after dilution of the reaction mixture to 10 ml. were unnecessary. Solutions deproteinized by the barium hydroxide-zinc sulfate (18) method were found suitable for these determinations. When glucose was present, this was determined independently and the glycol results adjusted, since 1 mole of glucose yields 1 mole of formaldehyde under the conditions described. The method is suitable when interfering substances are minimal or could be accurately determined, or, as in the cases cited above, when the added α-glycol is present in relatively overwhelming amounts.

obtained here melted at 70.2–70.6°. (Reported melting point for batyl alcohol, 70–71° (9).)

Respiration of Fragments of Starfish Diverticula—The arms were removed from starfish 6 inches in maximal diameter, and a cut was made along the entire length of the arm, between the rows of tube feet. The arm was opened, and the two diverticula were carefully removed, entire, from each arm. The tissue from one arm was more than sufficient for an average experiment on the Warburg machine.

The diverticula were thoroughly rinsed in filtered sea water which had been pasteurized at 70–80° for 30 minutes, cooled, and thoroughly oxygenated. Fragments of the tissue weighing approximately 100 mg. were cut, blotted, and accurately weighed on a Roller-Smith torsion balance before being introduced into the Warburg flasks, which contained pasteurized sea water. Measurements of the respiration of these fragments were made conventionally at 25°, the fat-free dry weights determined, and Q_{O_2} values calculated. Substrates were added to give a final concentration of 40 μmoles per ml.

Experiments with C^{14}-Labeled Glycerol and Acetate—α-C^{14}-Glycerol was prepared by the method of Gidez and Karnovsky (20) and 1-C^{14}-acetate by a standard procedure (Sakami *et al.* (21)). Approximately 3 gm. (accurately determined) of a starfish diverticulum cut into three to five pieces were placed in each flask, which contained 15 ml. of pasteurized oxygenated sea water and the C^{14}-substrate at a concentration of 40 μmoles per ml. The specific activities for glycerol and acetate were 2.20×10^5 and 3.45×10^5 c.p.m. per mmole, respectively. When both substrates were used, one radioactive and the other not, each was at a concentration of 40 μmoles per ml. The flasks were put in a water bath at 25°, oxygen was passed over the surface of the medium for 15 minutes with gentle shaking, and the flasks were then clamped. The incubation was carried out for 4 hours. pH determinations were made before and after the incubation and were 7.6 ± 0.2.

At the end of the incubation period, the tissue was removed from each flask, blotted dry, and washed for 2 minute periods in each of the following: 10 ml. of sea water, 10 ml. of sea water, 10 ml. of distilled water. The tissue was again blotted, ground with anhydrous magnesium sulfate, and extracted with about 25 ml. of chloroform in a small Soxhlet extractor for 24 hours. The total chloroform extract was then transferred to a glass-stoppered Erlenmeyer flask, and 15 ml. of cold distilled water were added. The layers of chloroform and water were very gently rotated with a magnetic stirrer for 45 minutes and allowed to stand still for 15 minutes, and the water was carefully withdrawn with a syringe. The process was repeated four times more. This washing had previously been shown to

remove all traces of C^{14}-glycerol or C^{14}-acetate added to non-radioactive starfish diverticulum fat in chloroform.

The chloroform solution was finally dried over anhydrous sodium sulfate overnight and filtered. The solids were thoroughly washed with fresh chloroform, and the washings were added to the filtrate. The chloroform was removed by distillation *in vacuo* and the fat recovered and maintained in a desiccator over paraffin wax, *in vacuo*, overnight. The total lipide was weighed, and a sample was counted.

The remaining fat was separated into neutral fat and phosphatides by acetone precipitation of the latter overnight in the ice box, with the aid of $MgCl_2$ (13). The phosphatides were centrifuged and washed with cold acetone, and the neutral fat was treated again as above to insure maximal phosphatide removal.

Saponification of Neutral Fat and Recovery of Unsaponifiable Fraction and Fatty Acids—The acetone was removed *in vacuo* from the neutral fat fraction and the fat weighed. When constant weight was attained, the fat was saponified in 5 ml. of 0.5 N NaOH (alcoholic) and the unsaponifiable fraction isolated essentially according to the Society of Public Analysts method (15). The alcoholic alkaline fraction, after removal of the unsaponifiable fraction, was made to pH 1 and exhaustively extracted with petroleum ether (b.p. 30–60°). The combined extracts containing fatty acids were washed with small volumes of water, and the petroleum ether was removed.

Treatment of Unsaponifiable Fraction—This fraction was dissolved in chloroform and heated with alcoholic digitonin to remove the sterols (22). After filtering the sterol digitonides and washing thoroughly with alcoholic digitonin, the filtrate and washings were combined and evaporated to dryness with gentle warming in a stream of nitrogen. The residue was extracted with dry ether and filtered. Excess digitonin remained in the residue, and the sterol-free unsaponifiable fraction passed into the filtrate. The ethereal filtrate was evaporated in a stream of N_2, and the substance obtained was oxidized in a 50 ml. non-protein nitrogen tube in ethyl alcohol with periodate, according to the method previously described (2), on a reduced scale. After destroying excess periodate with arsenite and adjusting the pH to 4.6, the solution was filtered through a small Celite cake to remove fatty substances. The cake was thoroughly washed and the formaldehyde in the combined filtrate and washings precipitated with dimedon. The formaldimedon was recrystallized and counted (melting point and mixed melting point with authentic formaldimedon, 189°). This represented the α-carbon of glycerol-ether glycerol. The Celite cake was sucked dry and extracted with 6 ml. of hot ethanol poured slowly through the pad. The clear extract was collected in a 15 ml. centrifuge

tube and cooled. Dinitrophenylhydrazone reagent (23) was added and the mixture stirred, warmed gently, and allowed to stand for several hours. The 2,4-dinitrophenylhydrazone of the fatty alcohol ether of glycolaldehyde precipitated as crystals and was centrifuged and recrystallized twice from diluted alcohol. The product was separated and dried, m.p. 74.6–75.2°. The melting point of the compound obtained as above from pure batyl alcohol was 75°. A mixed melting point was sharp and showed no depression. The reported melting point of the 2,4-dinitrophenylhydrazone of glycolaldehyde octadecyl ether is 73° (9).

Hydrolysis of Phosphatides and Recovery of Phosphatide Fatty Acids—The phosphatide fractions obtained above were dissolved in 1 ml. of EtOH, and 5 ml. of 6 N HCl were added with rotation of the flask. The mixture was heated 2 hours under reflux in a boiling water bath with frequent shaking. At the end of this time, 4 ml. of water were added, and the solution was transferred to a small separatory funnel and extracted four times, with a total of 60 ml. of petroleum ether. The petroleum ether extract was washed twice with 4 ml. of distilled water, which was added to the aqueous fraction. The petroleum ether solution was then shaken three times with 5 ml. of aqueous 0.5 N KOH solution each time. The alkaline extracts were combined and made to pH 1, and the fatty acids were re-extracted with ethyl ether, which was then removed under nitrogen, and the fatty acids were obtained.

Periodate Oxidation of Aqueous Fractions Obtained after Hydrolysis of Neutral Fats and Phosphatides. Neutral Fat—The aqueous fraction obtained after removal of the non-saponifiable fraction and fatty acids above was chilled, filtered, and oxidized with periodate after adjusting to pH 8 with bicarbonate. After 1 hour the excess periodate was destroyed by the addition of arsenite and HCl. The formaldehyde formed was distilled and the pH of the distillate brought to 4.6 by the addition of sodium acetate and HCl. The formaldehyde was precipitated with dimedon, centrifuged, twice reprecipitated from acetone by addition of water, and counted.

Phosphatides—The aqueous fraction after removal of fatty acids was chilled, filtered, made to pH 1, and shaken with 2 gm. of Dowex 1 in the sodium cycle (24) for 10 to 15 minutes. The mixture was centrifuged and poured through a fluted filter into a clean centrifuge tube and carefully neutralized with alkali. This treatment had been shown in exhaustive tests to remove serine and ethanolamine from the hydrolysate, which was ninhydrin-negative. The neutral aqueous fraction was then treated with $NaHCO_3$ and periodate as above. After the reaction was stopped, the pH was brought to 8, and the formaldehyde was distilled into a chilled centrifuge tube, where it was precipitated and reprecipitated as described.

Counting—All counting was carried out in a gas flow counter with high

efficiency (about 45 per cent) and low background (less than 10 c.p.m.) (25). Fats and fatty acids were counted as such on lens paper disks in the planchets. Formaldimedon and the 2,4-dinitrophenylhydrazone of the aldehyde-ether produced by periodate oxidation of glycerol ethers were plated directly. Under the conditions of counting in these laboratories, counts obtained on organic substances and on the $BaCO_3$ obtained by combustion of those substances agree well. These data will be presented elsewhere (26).

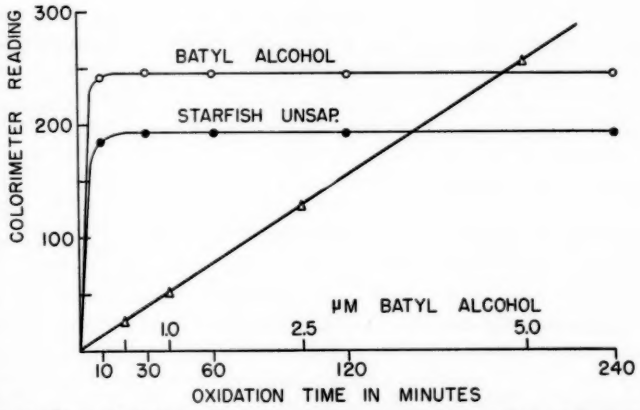

FIG. 1. Characteristics of the formation and determination of formaldehyde derived from α-glycerol ethers by oxidation with periodic acid. Conditions as described in the experimental section. The oxidation of batyl alcohol (○) and a starfish unsaponifiable fraction (●) was allowed to proceed for varying times, and the formaldehyde determined. Oxidation for 1 hour and with varying amounts of batyl alcohol (△).

RESULTS AND DISCUSSION

Determination of α-Glycerol Ethers—Fig. 1 illustrates the relationship of formaldehyde produced by an α-glycerol ether to time of reaction with periodate, when conditions are as described in the experimental section. It is clear that the reaction is complete within 30 minutes and a stable situation is attained. 1 hour has been adopted as the standard oxidation period. At this time the determination has been repeatedly found to conform to Beer's law, as is also shown in Fig. 1. With mannitol as the standard, samples of purified α-glycerol ether described in the experimental section were analyzed with the following results (calculated as batyl alcohol): starfish product (m.p. 68°), 106.2 per cent; (m.p. 70°), 100.0 per cent; tiger shark product (m.p. 70.4–70.6°), 108.1 per cent.

The mannitol used as standard assayed at 100.5 per cent by the gravimetric method of Reeves (27). When this mannitol was analyzed by the colorimetric method described above against a formaldehyde standard which had been independently assayed by the method of Yoe and Reid (28), a result of 98.6 per cent purity was obtained.

A number of organisms or tissues have been assayed for glycerol ethers by the colorimetric method described and by the more tedious gravimetric method, requiring large quantities, that had previously been employed

TABLE I

Glycerol Ether Contents of Some Unsaponifiable Fractions (Expressed As Batyl Alcohol)

Species	Common name	Source of fat	Fat*	Unsaponifiable†	Glycerol ethers	
					New method	Old method
			per cent	*per cent*	*per cent*	*per cent*
Loligo pealei	Squid	Digestive gland	10.5	3.9	6.3	0.0
Ommastrephes illecibrossus	Northern squid	" "	18.4	10.0	7.3	11.0
Asterias forbesi	Starfish	Diverticulum	9.5	9.5	61.8	63.9
Arbacia punctulata	Sea-urchin	Viscera	1.8	21.3	3.8	
Echinarachnius sp.	Sand-dollar	Whole animal		19.0	2.2	
Thyone briareus	Sea-cucumber	" "	1.1	10.1	11.0	11.5
Ciona sp.	Sea-squirt	" "	2.5	30.2	4.1	
Mustelus canis	Smooth dogfish	Liver	30	5.8	42.1	43.8
Carcharinus obscurus	Dusky shark	"	28	4.3	11.9	10.6
Galeocerdo cuvier	Tiger "	"	56	13.7	96.8	91.5
Clemmys guttata	Sea-turtle	"	13.2	7.6	3.7	

* Based on fresh weight of material in preceding column.

† Of fat in preceding column.

(2, 3). Table I summarizes the results. It may be seen that results by the two methods are in reasonable agreement.

By the method described here it is possible to obtain replicates that agree to within about 2 per cent; the accuracy is estimated to be between 3 and 5 per cent. Further, the method will give positive results with all α-glycols in which a hydroxyl is in a primary carbinol group. With the exception of some steroid compounds of mammalian adrenals and some long chain 1,2-glycols recently isolated from sheep wool fat (29), no interfering substances are known. The operations leading to the isolation of the fat and of the unsaponifiable fraction remove any water-soluble glycols, and the method is regarded as reliable, particularly in cases in which

α-glycerol ethers constitute a major component of the unsaponifiable fraction.

Experiments with Starfish—The starfish appeared to be a suitable animal for studies on the metabolism of α-glycerol ethers, since it is easily available and has a high content of these compounds occurring almost entirely as batyl alcohol (octadecyl α-glyceryl ether).

It is of interest to note that no "etherase" (catalyzing the hydrolytic split of the ether bond) could be detected in homogenates of starfish diverticula when a variety of glycol ethers and phenol ethers was employed. This was true also of shark pancreas and rat liver. This absence of hydrolytic splitting is in accord with thermodynamic expectation. Further, starfish maintained at a low oxygen tension for 2 weeks appeared no different with respect to their glycerol ether content. It would thus appear that these compounds are not formed in response to an environment of low oxygen tension.

It was, however, found that α-glycerol ethers accumulated in the starfish with age. The experiments were as follows:

The maximal diameter was used as an index of age, and batches of twenty to thirty starfish were accurately sorted into groups 4, 6 to 8, and 8 to 10 inches in maximal diameter. The diverticula were dissected, pooled in groups, and extracted. It was not found possible to remove the diverticula of animals 2 inches in diameter, and in this case the entire animal was extracted. It should be noted that previous analyses had shown that the exoskeleton fat contained no α-glycerol ethers; in the case of the 2 inch starfish, no glycerol ethers at all were detected in the whole animal. Fig. 2 illustrates the results and demonstrates the increase of α-glycerol ether ester content of the diverticulum fat with age.

Respiration of Fragments of Starfish Diverticula—The diverticulum of the starfish has been shown to be a very thin walled tube, greatly folded. The wall of this tube consists essentially of a row of tall columnar epithelial cells resting on a basement membrane, outside of which is a serosal layer. The thickness of the wall is about 250 μ (30). It was thought that fragments of such an organ might be suitable for metabolic experiments *in vitro*, since adequate diffusion of oxygen and nutrients into the tissue could occur.

Fig. 3 demonstrates the respiration of fragments of starfish diverticula under the conditions outlined in the experimental part. It is clear that the organ fragments continue to respire uniformly for lengthy periods, and that the respiration may be stimulated by the addition of glucose, glycerol, or acetate. The Q_{O_2} values were as follows: endogenous, 1.1; with glycerol, 1.7; with glucose, 2.2; with acetate, 1.9. These results indicated the feasibility of carrying out radio experiments *in vitro* with this system.

Incorporation of C^{14} into Starfish Lipide Fractions in Vitro—Fragments of

starfish diverticula were incubated with radioglycerol or radioacetate and the lipides extracted, fractionated, and counted as described in the experimental section.

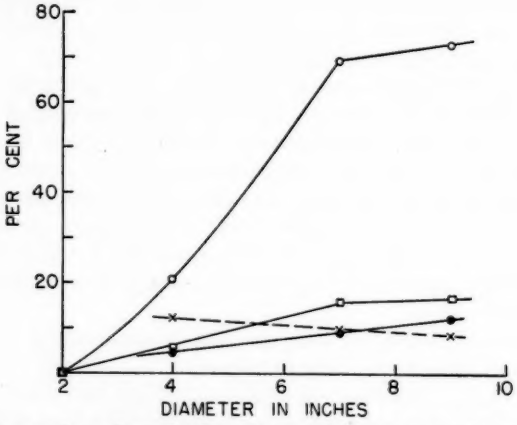

FIG. 2. Increase in α-glycerol ethers of starfish diverticula with age. Diameter used as an indication of age. ●, total fat, percentage of fresh tissue; ×, unsaponifiable fraction, percentage of total fat; ○, glycerol ethers(as batyl alcohol), percentage of unsaponifiable fraction; □, glycerol ether esters (as batyl dioleate), percentage of total fat.

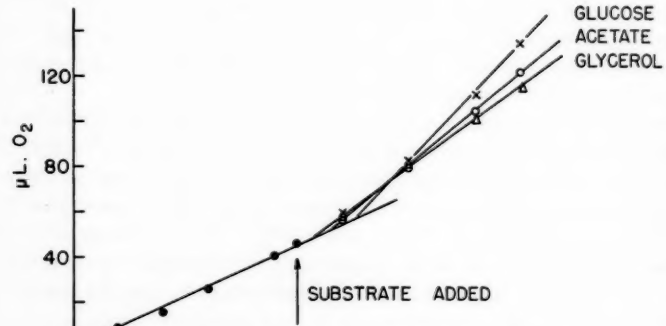

FIG. 3. Respiration of fragments of starfish diverticula. Conditions as described in the experimental section.

The following fractions were counted: (1) the fatty acids of the whole neutral fat fraction (including fatty acids originally esterified with glycerol ethers); (2) fatty acids of phosphatides; (3) the free primary carbinol group

of α-glycerol ethers, *i.e.* an α-carbon of the glycerol moiety, and consequently a measure of the activity of the glycerol moiety, since α-C^{14}-glycerol was employed as substrate; (4) the fatty alcohol ether of glycolaldehyde, obtained after the periodate oxidation of the glycerol ether fraction to obtain the fraction (3) above (by subtracting from the specific activity of this fraction the activity of the residual α-carbon of the glycerol moiety, the specific activity of the fatty alcohol moiety (calculated as octadecanol) could be obtained); (5) formaldehyde produced by periodate oxidation of the aqueous hydrolysis fractions from both neutral fats and phosphatides.

TABLE II

Incorporation of C^{14} into Lipide Fractions of Starfish Diverticulum

Total fat set at 100 c.p.m. per mg. after normalization of the activity administered; results as counts per minute per millimole $\times 10^{-3}$, $\pm$ the standard error of the mean.

Substrate	No. of experiments	Fatty acids*		Fatty alcohol†	Formaldehyde		
		Neutral fat	Phosphatide	Glycerol ethers	Neutral fat hydrolysate	Phosphatide hydrolysate	Glycerol ethers
Glycerol‡	6	7.3 ±1.5	14.2 ±2.3	17.4 ±4.5	8.9 ±1.3	33.4 ±6.1	5.7 ±2.1
" ‡ and acetate	1	3.6	2.2	9.5	17.0	57.0	3.7
Acetate‡	4	26.0 ±2.9	52.4 ±4.0	29.2 ±11.1	0.2 ±0.15	1.7 ±1.1	0.0
" ‡ and glycerol	1	35.3	20.0	15.9	0.0	0.0	0.0

* Calculated on the basis of stearic acid as the fatty acid.

† Calculated as octadecanol (*i.e.*, the C_{18}-alkyl moiety of batyl alcohol).

‡ Denotes radioactive substrate.

The results are reported in Table II. For convenience, the activity of the total fat has been set at 100 c.p.m. per mg., and the other fractions are expressed proportionally. This minimizes differences among individual starfish due to dietary status and other factors.

The results show the following. (1) The carbon of both acetate and glycerol is incorporated into the fatty acids of neutral fat and phosphatide. The fatty acids of the latter were, when only one substrate was present, about twice as active as those of the neutral fat fraction. (2) Activity was significantly incorporated into the long chain alkyl moiety of the glycerol ethers when either radioglycerol or radioacetate was present. (3) When radioglycerol was the substrate, activity appeared in the α-carbon of the glycerol moiety of the glycerol ethers, but not when radioacetate was the

substrate. This is true also for the formaldehyde obtained by periodate oxidation of the aqueous hydrolysis fractions of neutral fat and phosphatide, for which the slight activity observed in the case of experiments with radioacetate cannot be regarded as significant. This formaldehyde might represent with reasonable accuracy the α-carbons of triglyceride and phosphatide glycerol, since in the latter case serine and ethanolamine had been removed. This has been found to be true in the rat (31). However, in a fat as imperfectly characterized as that examined here, such an assumption would not be warranted.

The specific activity of the fatty alcohol moiety of the glycerol ethers was calculated as mentioned above by correction of the measured specific activity of the glycolaldehyde alkyl ether for the activity of the remaining glycerol α-carbon. Such a calculation assumes equality of labeling of the 2 α-carbons of glycerol-ether glycerol (which is true of the administered α-C^{14}-glycerol) and low randomization of counts into the β-carbon of glycerol-ether glycerol. Randomization is, in the case of the rat, very low in such short term experiments (31) and might be expected to be so in the much more slowly metabolizing system used here.

The incorporation of radiocarbon into α-glycerol ether moieties at rates comparable to those observed in the more commonly studied lipide fractions indicates that the starfish does not merely accumulate these compounds from its food. The mechanism of synthesis and splitting of the ether bond is as yet not understood, but the tissue studied here appears a promising source of the enzymes involved.

SUMMARY

1. A method has been devised for the colorimetric determination of α-glycerol ethers in the unsaponifiable fractions of fats, and a number of different fats have been analyzed by this method.

2. The starfish has been used as an experimental animal. No hydrolytic mechanism of splitting ethers was detected, nor was any response to oxygen tension of the environment discernible. Increasing age (as determined by size) could, however, be strikingly correlated with the magnitude of α-glycerol ether occurrence.

3. Following satisfactory respiration experiments, exposure of pieces of starfish diverticula to C^{14}-acetate or C^{14}-glycerol indicated that both these substrates were incorporated into α-glycerol ethers to an extent comparable to that found for other lipide entities.

4. Although glycerol carbon was incorporated into both glycerol and fatty alcohol moieties of α-glycerol ethers, acetate carbon appeared only in the fatty alcohol moiety.

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STUDIES ON LEUCINE BIOSYNTHESIS IN YEAST*

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Studies with microorganisms grown on labeled substrates have yielded a substantial amount of information on the mechanism of biosynthesis of various amino acids. Little attention has, however, been paid to the origin of the amino acid leucine and hence its origin has remained largely obscure. It was earlier reported from this laboratory (1, 2) that, in yeast cultured on glucose and supplied with C^{14}-acetate, the amino acids of the cellular proteins contain isotopic carbon at three different levels and may be grouped accordingly: phenylalanine and tyrosine with insignificant concentrations of acetate carbon, a large number of the amino acids which contain intermediate levels of isotope, and a group consisting of lysine, proline, arginine, glutamic acid, and leucine with C^{14} levels approaching those of the yeast lipides. It was suggested that only for the synthesis of this last group of amino acids, which includes leucine, was acetate utilized as a direct carbon source.

The present report deals in greater detail with the contribution of the 2 carbon atoms of acetate carbon to leucine biosynthesis, and also with the utilization of 1-C^{14}-glucose in the same process. It includes data on the distribution of acetate and glucose carbon which is obtained on degradation of the leucine molecule.

EXPERIMENTAL

From earlier experiments (2, 3), three different hydrolysates of yeast protein were available: (1) from a batch of yeast (*Saccharomyces cerevisiae*) grown on a medium containing non-isotopic glucose and 1-C^{14}-acetate (Experiment A); (2) from yeast grown on non-isotopic glucose and 1-C^{13}-, 2-C^{14}-acetate (Experiment B); and (3) one from cells grown in the presence of 1-C^{14}-glucose and 1-C^{13}-acetate (Experiment C). In a fourth experiment (Experiment D) non-isotopic glucose and 1-C^{14}-acetate were the carbon sources. The composition of the media, the culture conditions, and the isolation of the yeast protein are described in an earlier publication (2).

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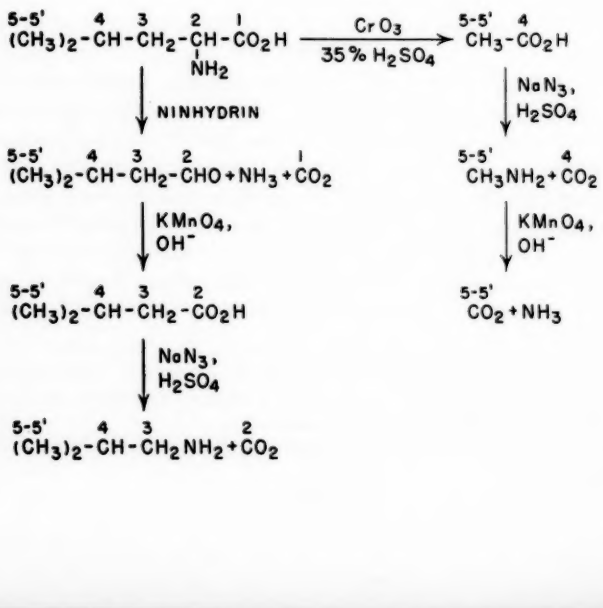
Isolation of Leucine—Hydrolysates from about 10 gm. of yeast were concentrated to a volume of 10 ml. and placed on a column of Dowex 50 (200 to 400 mesh), 4.2 × 80 cm., according to the procedure of Moore and Stein (4). On elution of the amino acids with 2.5 N HCl, leucine failed to emerge from the column as a single peak; it was contaminated with methionine, isoleucine, and cystine. The first quarter of the "leucine" fraction, which consists mainly of methionine and isoleucine, was discarded, and the remainder rechromatographed on a fresh column of Dowex 50. The eluates were brought to dryness *in vacuo*, redissolved in water, and the amino acid recrystallized from a solution that had been adjusted to pH 5.8 and contained 20 per cent ethanol. After recrystallization, the amino acid gave a single ninhydrin-positive spot with an R_F value of 0.51 in the solvent system described by Block (5). C^{14} activity was located in the same area. When tested microbiologically with *Lactobacillus arabinosus*, the amino acid was assayed as pure leucine within the limits of the experimental error (±5 per cent) (6, 7). By the same technique the content of isoleucine was shown to be less than 0.1 per cent. Absence of methionine was established by a negative nitroprusside test (8), and contamination by purines and pyrimidines was ruled out by the lack of absorption in the ultraviolet region.

Degradation of Leucine—After dilution of the isotopic leucine with unlabeled amino acid to the desired level of radioactivity, isotope concentrations were determined by combustion to CO_2. The procedures employed

DEGRADATION OF LEUCINE



for the degradation of leucine are summarized in the accompanying diagram.

An aliquot of leucine was decarboxylated (9) with ninhydrin to yield C_1 as CO_2; isovaleraldehyde, the second product of the decarboxylation, was distilled in a stream of nitrogen, condensed in a dry ice trap, and oxidized with alkaline permanganate (10 mmoles of $KMnO_4$ and 0.33 mmole of KOH in 100 ml. of water) for 5 minutes at 40°. After cooling of the solution, the excess permanganate was destroyed by addition of oxalic and sulfuric acids, and isovaleric acid was recovered from the solution by steam distillation. The acid, after purification by chromatography on a silica gel column, according to Marvel and Rands (10), was obtained in 48 to 50 per cent yield from leucine. The compound had the correct Duclaux constant, and its *p*-bromophenacyl ester (m.p. 66.5°) had a specific activity which agreed with the value calculated from that of leucine minus the carboxyl carbon of the amino acid.

Carbon atom 2 of leucine was obtained as CO_2 by the Schmidt degradation of isovalerate according to the procedure described by Phares for propionic acid (11). After the reaction mixture had been freed of CO_2, the pH was raised to 12 and the isobutylamine distilled into a trap containing 2 N H_2SO_4. For isotope analysis, the phenylthiourea derivative of isobutylamine (m.p. 81°) was prepared. Another aliquot of leucine was subjected to chromic acid oxidation according to the Kuhn-Roth method (12), yielding 0.7 mole of acetic acid per mole of amino acid. The identity of the fatty acid was established by its Duclaux constant and by chromatography on silica gel. For isotope analysis the acetic acid was converted either to silver acetate or to 8-methylbenzimidazole (m.p. 177–178°) (13). Decarboxylation of acetic acid by the Schmidt reaction (11) yielded CO_2 and methylamine, which correspond to carbon atoms 4, and 5 and 5′, respectively, of leucine. Methylamine was oxidized to CO_2 by alkaline permanganate.

The isotope concentrations of carbon atoms 4, 5, and 5′ were determined by a second independent method which proved less tedious than the one just described because it obviated the need of isolating pure leucine from the protein hydrolysate. The crude leucine fraction (containing also isoleucine and methionine), obtained after a single passage of the protein hydrolysate through Dowex 50, was subjected to ninhydrin treatment, and the resulting mixture of aldehydes was trapped in a 1.2 per cent solution of 2,4-dinitrophenylhydrazine (DNP) in 2 N perchloric acid (14). The precipitated hydrazones were filtered, washed with cold 70 per cent ethanol, and dried. They were then dissolved in warm carbon tetrachloride and chromatographed on alumina (15) (Merck, "Suitable for chromatographic absorption"). When the column was developed and eluted with petroleum

ether (b.p. 30–60°) containing 5 per cent methyl acetate, the absorbed hydrazones separated into several bands. The two fastest moving components were identified as the DNP derivatives of 2-methylbutyraldehyde (m.p. 133.5–134.5°) and of isovaleraldehyde (m.p. 119–120°) respectively. That the two hydrazones had been effectively separated was also shown by their specific activities. The hydrazone of 2-methylbutyraldehyde (from isoleucine) had a specific activity of 770 c.p.m. and that of isovaleraldehyde (from leucine) of 10 c.p.m. (Table I, Experiment A).

The DNP derivative of isovaleraldehyde was subjected to Kuhn-Roth oxidation as described above for leucine, and yielded 0.54 mole of acetate per mole of hydrazone. The specific activities obtained by the two methods, *i.e.* by direct oxidation of leucine and by oxidation of the hydrazone of isovaleraldehyde, were 125 and 117 for C_4 and 1910 and 1750 for C_5 and $C_{5'}$, respectively.

TABLE I

Experiment A, Incorporation of 1-C^{14}-Acetate into Leucine of Yeast Protein

Products	Specific activity, c.p.m.
Leucine	8,380
C_1 of leucine	50,780
2,4-Dinitrophenylhydrazone of isovaleraldehyde	10
" " α-methylbutyraldehyde	770

Isotope Analyses—All the samples, except those which were obtained as CO_2 on degradation, were subjected to combustion, and the CO_2 was precipitated as $BaCO_3$ and counted in a gas flow counter for a sufficient length of time to reduce the probable error to less than 5 per cent. The counts, which are given as counts per minute of infinitely thick samples of $BaCO_3$, were corrected for infinite thickness according to Reid (16). C^{13} analyses were carried out according to Rittenberg (17).

Results

Utilization of Acetate—Data on the utilization of the 2 carbon atoms of acetate and of 1-C^{14}-glucose for leucine synthesis in yeast are given in Tables I to IV. When 1-C^{13}- or 1-C^{14}-acetate was the labeled source, nearly all of the isotopic carbon in leucine could be recovered in the carboxyl group of the amino acid. Thus, in Experiment B, Table II, leucine contained 1.13 atom per cent excess C^{13} and its carboxyl group 6.4 per cent or 94 per cent of the total C^{13} in the molecule; in Experiment C, Table III, the corresponding value was 109 per cent. Moreover, analysis of the DNP derivative of isovaleraldehyde (all carbons but C_1), Table I, showed that acetate carbon was exclusively incorporated into C_1 of leucine.

When 2-C^{14}-acetate was the precursor, label appeared predominantly at carbon atom 2 of leucine. In the two experiments (Tables II and IV), C_2 of leucine accounted for 67 and 74 per cent, respectively, of the total C^{14}

TABLE II

Experiment B, Utilization of $C^{14}H_3C^{13}O_2H$ by Yeast

	Specific activity		$CH_3:CO_2H$
	C^{14}	C^{13}	
	C.p.m.	Atom per cent excess	
Initial acetate*	22,800	10.4	1.00
Final acetate*	1,270	0.70	0.83
Fatty acids*	7,830	3.66	0.98
Leucine	80	1.13	1.20
" C_1	97	6.4	0.85
" C_2	11,850	0	
" $C_{4,5}$	194		
" C_3 (calculated)	5,340		

* Data from Gilvarg and Bloch (2).

TABLE III

Experiment C, Yeast Grown in Presence of 1-C^{14}-Glucose and 1-C^{13}-Acetate

	C^{14}, c.p.m.	C^{13}, atom per cent excess
Glucose	810	0.0
Acetate, initial*	0	10.4
" final*	800	3.15
Leucine	750	1.50
" C_1	32	9.8
" C_2	910	
" $C_{4,5}$†	980	
" C_4†	121	
" $C_{5,5'}$†	1830	
" C_3 (calculated)	−228	

* Data from Gilvarg and Bloch (3).

† Wherever the isotope concentration of a compound was determined by preparation and analysis of more than one derivative, the average specific activity is reported here.

in the amino acid. Of the remainder, 30 and 13 per cent, respectively, appeared to be located at C_3. However, the values for C_3 must be regarded with some reservation since they were calculated by difference (6 times the specific activity of leucine minus the sum of the specific activities of C_5,

$C_{5'}$, C_4, C_2, and C_1). In the synthesis of leucine the methyl carbon of acetate is utilized to a somewhat greater extent than the carboxyl carbon, since for the whole molecule the ratio of the relative isotope concentrations (methyl to carboxyl ratio) is 1.20. In the conversion of the methyl carbon of acetate to C_2 of leucine, the dilution of isotope is nearly the same as in the conversion of acetate carboxyl to C_1 of the amino acid (methyl to carboxyl ratio of 0.85). Moreover, since no significant quantity of acetate methyl appears at C_1 of leucine, nor of acetate carboxyl at C_2, these data suggest that carbon atoms 1 and 2 of leucine are derived from acetate as a unit.

TABLE IV

Experiment D, Yeast Grown in Presence of $C^{14}H_3CO_2H$

	Specific activity, c.p.m.
Initial acetate*	19,500
Final acetate*	7,200
α-Carbon of alanine*	675
β-Carbon " " *	600
Leucine	3,880
" C_1	151
" C_2	17,330
" $C_{4,5}$†	700
" C_4	735
" $C_{5,5'}$†	685
" C_2 (calculated)	2,990

* Data from Gilvarg and Bloch (2).

† Average specific activity determined by analysis of more than one derivative or degradation product.

Fatty acids are synthesized by condensation of acetyl units, and hence the isotope concentration of the fatty acid component of the cellular lipides should reflect the average isotope concentration of acetate during the growth period. In Experiment B, Table II, the fatty acids had 7830 c.p.m. and 3.66 atom per cent excess C^{13}, or, assuming that C^{14} was present at even-numbered carbon atoms only, and C^{13} at odd-numbered carbon atoms, the acetyl precursor should have contained 15,660 c.p.m. in the methyl carbon and 7.3 atom per cent excess C^{13} in the carboxyl carbon. The values found for C_1 and C_2 of leucine, namely 6.8 per cent C^{13} and 11,850 c.p.m., compare favorably with those calculated for the acetyl precursor available for synthetic reactions. Of interest in this connection is the distribution of the carbon atoms of acetate in lysine which has been observed by Strassman and Weinhouse (18) and also in this laboratory,[1] and which has been the

[1] Gilvarg, C., and Bloch, K., unpublished results.

basis of the suggestion that the carbon skeleton of lysine (18) is formed by the condensation of an acetate unit with a dicarboxylic acid. Lysine isolated from the protein hydrolysate of Experiment B contained 17,000 c.p.m. at C_2 and 8.53 atom per cent excess at the carboxyl carbon[1] corresponding to a methyl to carboxyl ratio of about 1.

The data obtained with 1-C^{14}-glucose and 1-C^{13}-acetate in Experiment C (Table III) are also consonant with the proposal that carbons 1 and 2 of leucine are derived directly from an acetate unit. It may be assumed that the 1-C^{14}-glucose which is added to the medium is metabolized by way of 2-C^{14}-acetate. On analysis of the recovered acetate it is found that the specific activity of the methyl group of acetate increases from an initial value of 0 to 1600 c.p.m. at the end of the growth period. At the same time, the C^{13} concentration of the carboxyl group of acetate decreases from 20.8 to 6.3 atom per cent excess as a result of dilution by glucose carbon. The isotope concentrations of carbons 1 and 2 of leucine, 910 c.p.m. at C_2 and 9.7 atom per cent excess at C_1, lie about half way between the "initial" and the "final" acetate values.

Methyl carbons of acetate contribute to a slight but significant degree to the synthesis of all 3 carbon atoms of the isopropyl group of leucine. It is worth noting that the specific activities of C_4, C_5, and $C_{5'}$ of leucine are of the same magnitude as those of the α- and β-carbon atoms of alanine, which in turn differ only slightly from each other (Table IV). This fact suggests, as will be discussed later, a common origin for the isopropyl portion of leucine and for alanine.

Utilization of 1-C^{14}-Glucose—When 1-C^{14}-glucose was the carbon source, C^{14} was incorporated predominantly at carbon atoms 2 and 5 or 5′ of leucine (Table III). The level of C^{14} is twice as high in the 2 isopropyl carbons as it is at C_2, indicating that C_5 and $C_{5'}$ are more directly derived from glucose than the remainder of the leucine molecule. It is conceivable that in the course of leucine biosynthesis only one of the two carbons which become C_5 and $C_{5'}$, and which are inseparable analytically, contain C^{14}. In this case the specific activity of C_5 (or $C_{5'}$) could have been 2×1830, or 3660 c.p.m. This value is appreciably lower than the 4800 c.p.m. present at C_1 of the labeled glucose which was added to the growth medium, and therefore argues against a direct utilization of glucose. It is still compatible, however, with the view that a singly labeled triose derived from carbon atoms 1, 2, and 3 of glucose, such as dihydroxyacetone phosphate, was a precursor of the isopropyl moiety.

In the alternative event that in a leucine precursor the 2 carbon atoms which furnish 5 and 5′ of the amino acid had both been labeled, a glycolysis product having C^{14} at two positions in excess of 1830 c.p.m. would have to be postulated. For example, free 1,3-C^{14}-dihydroxyacetone would have a specific activity of 2400 c.p.m. at each labeled carbon atom as a result of

randomization, and would therefore meet these requirements. On the other hand, isomerization of the glycolysis products would yield a mixture of trioses having a specific activity of 2400 c.p.m. at the β-carbon atoms or an average specific activity of 800 c.p.m. for all 3 carbon atoms, a value which is too low to account for the C^{14} content of the isopropyl group of leucine (average specific activity, 1260 c.p.m.).

There is evidence, however, which argues altogether against the utilization of a triose as an immediate precursor of the isopropyl portion of leucine. It may be recalled that, in yeast cultured on normal glucose and C^{14}-acetate, the carbon atoms of acetate fail to be incorporated into the cellular carbohydrate (19). Under these conditions acetate carbon is converted to alanine, but is not utilized for the synthesis of the side chains of tyrosine and phenylalanine. These findings, coupled with the evidence that the side chains of the aromatic amino acids are derived from 3-carbon units of glycolysis (3), tend to show that in yeast which is grown under specified conditions the reversal of the glycolytic reactions occurs at a slow rate and, more specifically, that the limiting step involves intermediates above the oxidation level of pyruvate. Since in the experiments with 2-C^{14}-acetate, C_4, C_5, and $C_{5'}$ of leucine contained C^{14} in considerable amounts, though little if any C^{14} was introduced into glycolytic intermediates, products of glycolysis which are more reduced than pyruvate would have to be ruled out as precursors of the isopropyl portion of leucine. In this case pyruvic acid or the members of the tricarboxylic acid cycle are the only possible carbon sources which remain. From the arguments just presented, it will be clear that pyruvic acid derived from 1-C^{14}-glucose should have the following isotopic composition: $CH_3\overset{2400}{C}O—COOH$. Hence, a single pyruvate molecule could furnish only part of the isopropyl group. It is equally difficult to conceive that dicarboxylic acids derived from 1-C^{14}-glucose would contain C^{14} at alternate positions ($—C\overset{x}{—}C\overset{x}{—}C—$) in sufficient concentration to serve as a precursor for this moiety of leucine.

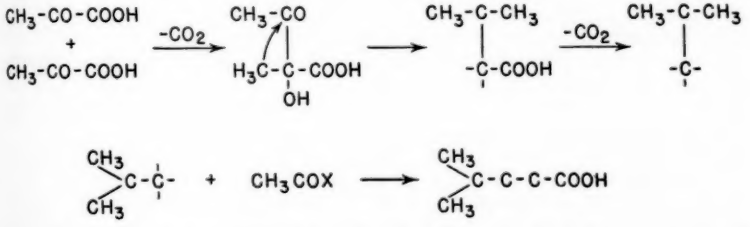
In the discussion of the incorporation of 2-C^{14}-acetate into leucine, attention was drawn to the similarity of isotope concentrations at the α- and β-carbon atoms of alanine and of the isopropyl carbons of leucine, and a common origin such as pyruvate was suggested. In view of the requirements which have been set above for the leucine precursor from glucose, it is clear that more than 1 molecule of pyruvate would have to participate in the synthesis of the isopropyl group, for example 2 β-carbon atoms of pyruvate furnishing C_5 and $C_{5'}$.

A mechanism capable of accounting for the utilization of various labeled precursors in valine biosynthesis has been proposed by Strassman *et al.*

(20). Condensation of 2 molecules of pyruvate to acetolactate, followed by a pinacol type rearrangement to form dimethylpyruvic acid, is the salient feature of the scheme suggested by these investigators. It should also be mentioned here that Abelson, on the basis of studies with *Escherichia coli*, has suggested the valine precursor α-ketoisovalerate as a source of all but C_1 and C_2 of the carbon skeleton of leucine (21). For purposes of comparison, the distribution of the carbon atoms of acetate and of glucose in the two branched chain amino acids from Experiments A and C (Tables III and IV) is given in the accompanying diagram; the data for valine are those obtained by McManus in this laboratory (22).

	Precursor 2-C^{14}-Acetate					Precursor 1-C^{14}-Glucose				
Valine	$(CH_3)_2$	— C —	C —	C		$(CH_3)_2$	— C —	C —	C	
	856	1240	697	126		1560	846	0	55	
	685	735	2990	17,330	151	1830	121	(0)	910	32
Leucine	$(CH_3)_2$	— C —	C —	C —	C	$(CH_3)_2$	— C —	C —	C —	C

$C_{4,4'}$ of valine and $C_{5,5'}$ of leucine have specific activities which agree moderately well, but the remaining carbon chains of the two amino acids show little resemblance in isotope distribution. For this reason the mechanism proposed for valine biosynthesis can be applicable to leucine only in a very restricted sense. The same principle, namely formation of an iso-



propyl group by interaction of 2 molecules of pyruvate and rearrangement of the carbon chain, may nevertheless be involved. Since there is considerable evidence that carbon atoms 1 and 2 of leucine are derived from an acetyl unit, it may be suggested that the product of rearrangement is converted to an intermediate having the carbon skeleton of isobutyric acid before condensation with the acetyl unit takes place.

According to this scheme, the β-carbon atoms of pyruvate provide C_5 and $C_{5'}$ of leucine and the α-carbons of pyruvate would be the source of C_4 and C_3. The marked difference in isotope concentration at C_4 and C_3 of leucine in the experiments with 2-C^{14}-acetate is the only experimental

result which seriously disagrees with the proposed mechanism. However, as has been mentioned, the value assigned to C_3 may be considerably in error, since it was obtained by calculation rather than by direct analysis.

SUMMARY

Leucine was isolated from yeast grown on media containing 1-C^{14}-acetate, 2-C^{14}-acetate, or 1-C^{14}-glucose, with glucose as the principal carbon source in all cases. The data obtained on degradation indicate that C_1 and C_2 of leucine are derived from an acetyl unit. The possibility is discussed that the remaining carbon chain of leucine is formed from 2 molecules of pyruvic acid.

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THE ACETYLATION OF 2-AMINOFLUORENE AND THE DEACETYLATION AND CONCURRENT REACETYLATION OF 2-ACETYLAMINOFLUORENE BY RAT LIVER SLICES*

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In a previous spectrophotometric study of the metabolism of the carcinogen 2-aminofluorene (AF) by the rat, evidence was presented that a fraction of the injected compound occurred in the tissues in a conjugated form as indicated by an increase in diazotizable amine after acid hydrolysis (1). It appeared that the liver was especially active in conjugating AF since the concentration of the conjugated compound in this organ increased approximately 2-fold from 4 to 12 hours after injection of the carcinogen. On the other hand, the concentrations of conjugated material declined markedly in the other internal organs and the carcass. These data suggested that AF might be acetylated by the rat and, in particular, by the liver. The present study is concerned with the identification and quantitative estimation of 2-acetylaminofluorene (AAF) and 2-diacetylaminofluorene (DAAF) as metabolites of AF in rat liver slices. By use of acetate-1-C^{14}, information concerning the nature of the acetylating agent and of the apparent reversibility of the acetylation reaction in rat liver was also obtained.

EXPERIMENTAL

Animals, Tissue Preparations, Incubation Media, and Synthesis of Unlabeled and Labeled Compounds—The experimental details regarding animals, tissue preparations, and incubation systems were essentially the same as those reported in a previous study (2). Unlabeled AF (3), AAF (4), DAAF (5), and 2-hydroxy-7-acetylaminofluorene (2-OH-7-AAF) (6) were prepared by published methods. AF-9-C^{14} (7), m.p. 127–128°,[1] had a specific radioactivity of 2070 c.p.m. per γ (3.12×10^7 c.p.m. per mg. of carbon 9). Radiochemical purity of the compound was shown by a single radioactive peak ($R_F = 0.89$) on scanning of paper chromatograms (8) which had been developed, by ascending chromatography, in *n*-butanol

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[1] All melting points were taken on a Fisher-Johns melting point apparatus and were corrected to reference standards.

saturated with 3 M ammonium hydroxide. Likewise, paper electrophoresis of the material with 20 per cent acetic acid as electrolyte gave a single radioactive peak (mobility = 4.5×10^{-5} cm. per second per volt per cm.) (9). AAF-9-C^{14} (7), m.p. 197–198°, had a specific radioactivity of 1410 c.p.m. per γ (2.62×10^7 c.p.m. per mg. of carbon 9). The compound was not contaminated with radioactive AF, since paper electrophoresis of 150 γ gave no radioactive peak for AF. The lower limit of sensitivity of the scanning device is 200 c.p.m., which is equivalent to 0.2 γ of AF. Sodium acetate-1-C^{14} was prepared by distilling 84 mg. of acetyl chloride-1-C^{14} (1 mc.)[2] *in vacuo* and trapping the distillate on ice cooled externally by liquid nitrogen. The labeled acetic acid was titrated to pH 9.0 (glass electrode) with 0.972 N sodium hydroxide. The final solution contained 8.7 mg. of sodium acetate-1-C^{14} per ml. (98.8 per cent yield). The sodium acetate had a specific radioactivity of 1270 c.p.m. per γ (2.42×10^6 c.p.m. per mg. of acetyl).

Chromatographic Procedures—For the identification of AAF-9-C^{14} the supernatant liquid of the incubation mixture was separated from the slices by filtration. The filtrate was acidified with concentrated hydrochloric acid to pH 1 (Hydrion paper) and continuously extracted with diethyl ether for 12 hours. The ether was evaporated and the residue dissolved in ethanol. Aliquots of the ethanol solution were applied to paper strips (Whatman No. 1) 17 cm. long and 3.8 cm. wide. The papers were developed at 28° by the ascending technique with hexane-benzene (2:1) saturated with distilled water as a solvent (Fig. 1).

For identification of acetyl-labeled AAF and 2-OH-7-AAF the supernatant liquids of the incubation mixtures were separated from the slices by filtration and poured into 10 volumes of acetone. The slices were homogenized with distilled water and the homogenates poured into 10 volumes of acetone. The mixtures were centrifuged and the protein precipitates washed three times with 25 ml. of acetone. The aqueous acetone solutions were combined and evaporated to dryness *in vacuo* at room temperature. The residue was treated with boiling ethanol, and aliquots of this solution were applied to paper strips. For the detection of AAF the papers were chromatographed in the butanol-ammonia-water system after they had been subjected to electrophoresis (9) (Fig. 4). AAF was also detected by scanning of papers developed in the hexane-benzene-water system. In this solvent system 2-OH-7-AAF did not migrate. Advantage was taken of this fact for the separation of AAF and 2-OH-7-AAF as follows. Aliquots of the ethanol solution were applied to the geometric centers of papers 32 cm. long and 3.8 cm. wide. The papers were then immersed for development by ascending chromatography in the hexane-benzene-water system

[2] Acetyl chloride-1-C^{14} was obtained from Tracerlab, Inc., Richmond, California.

to within 2.0 cm. of the spots of application. After drying the developed papers, the region which contained the radioactive AAF was cut off 2.5 cm. above the spot of application. The remaining portion of the paper was inverted and chromatographed in the butanol-ammonia-water system by the ascending technique. The papers were then scanned for radioactivity and subsequently sprayed with diazotized sulfanilic acid (9) (Fig. 4). The color peak of the sprayed papers was determined with a densitometer (Welch densichron) equipped with a Wratten red F filter.

Carrier Experiments—Prior to the addition of the appropriate carrier the soluble proteins of the supernatant liquids and the slice proteins were precipitated with acetone as described for the chromatographic identification of acetyl-labeled AAF. The combined aqueous acetone solutions were then adjusted to a suitable volume, and the radioactivity of the solution was determined. Unlabeled AF, m.p. 127–128°, AAF, m.p. 197–198°, or DAAF, m.p. 134–135°, dissolved in 10 to 20 ml. of acetone, was then added, and the solutions were thoroughly mixed. The acetone was removed by vacuum distillation at room temperature. For the isolation of AAF or DAAF, the aqueous suspensions were acidified to pH 1 with concentrated hydrochloric acid. For the isolation of AF, they were made alkaline to pH 10 with concentrated ammonium hydroxide. The mixtures were then continuously extracted for 24 to 36 hours with diethyl ether. The ether extracts were washed with distilled water, and the ether was evaporated. The recovered carrier compounds were crystallized to constant specific radioactivity from appropriate solvents listed and Tables I and III and Figs. 2 and 3. Details of additional purification procedures by adsorption chromatography or sublimation are described in Figs. 2 and 3. As a further proof of purity of the isolated carrier compounds which had been crystallized to constant specific activity from two different solvents, the following derivatives were prepared with no change of the specific radioactivity. AAF-9-C^{14} or DAAF-9-C^{14} was hydrolyzed to AF-9-C^{14} by refluxing for 1 hour in a 1:1 ethanol-concentrated hydrochloric acid solution. AF-9-C^{14} was obtained by neutralizing the solution with concentrated ammonium hydroxide. Acetyl-labeled AAF was acetylated to DAAF by applying the procedure for the diacetylation of AF (5). AF-9-C^{14} was acetylated to AAF-9-C^{14} (4).

Protein Binding of Radioactivity—The acetone powders obtained from the homogenized slices were treated as described previously (2).

Radioactivity Measurements—Duplicate samples of compounds, tissues, and extracts were oxidized by the wet combustion technique, and the radioactivities of the barium carbonate precipitates were measured in a windowless gas flow counter (10). Counting times were of sufficient length to reduce the standard error of counting to 5 per cent or less. Samples with

activities of 30 c.p.m. or less were counted with a standard error of 10 per cent (11). In the experiments with sodium acetate-1-C^{14} the effluent gases were passed through two absorbing traps connected in series and containing carbonate-free 2.5 per cent sodium hydroxide (7). The recovery of the radioactivity was 90 to 110 per cent. In the experiments with compounds labeled in the 9 position the recoveries were 97 to 112 per cent.

Results

The ability of rat liver slices to acetylate AF was shown qualitatively by the chromatographic detection of AAF-9-C^{14} in an extract prepared from an incubation mixture of liver slices with AF-9-C^{14} as substrate (Fig. 1). Additional proof for this reaction and an estimate of its extent were furnished by the carrier experiments of Table I. 23.8 per cent of the added AF-9-C^{14} was acetylated in 4 hours in Experiment 1. In another experiment, carried out under identical conditions, but not reported in Table I, 19.0 per cent of the available AF-9-C^{14} was acetylated. In this case, the carrier compound was crystallized to constant specific radioactivity and proved to be pure by the constant solubility test (12). When unlabeled sodium acetate was added to the incubation mixtures (Experiment 1A, Table I), a marked stimulation of the acetylation of AF was observed. It will be noted that the increase of acetylation after the addition of small quantities of acetate (0.89 mg. per flask) occurred in the presence of a high concentration of glucose (72 mg. per flask). Under the experimental conditions glucose will undoubtedly supply precursors for acetyl groups via the tricarboxylic acid cycle. For example, Topper and Stetten have recently shown that succinate-1,4-C^{14} furnishes acetyl groups for the acetylation of sulfanilamide (13). The conditions under which stimulation of the acetylation of AF occurred in the present experiments suggested that acetate (presumably after activation to acetyl coenzyme A, as more fully discussed below) was the principal and immediate acetyl donor. More direct evidence for this rôle of acetate was provided by the isolation of acetyl-labeled AAF after incubation of liver slices with AF and acetate-1-C^{14} (Experiment 2, Fig. 2). Control experiments with slices which were heated in an autoclave at 15 pounds pressure per sq. in. and 120° for 20 minutes prior to incubation with AF and acetate-1-C^{14} gave no chromatographic evidence for the presence of acetyl-labeled AAF, demonstrating the metabolic nature of the acetylation reaction. The participation of endogenous acetyl groups in the reaction is apparent from the following considerations. With the method of calculation of Table I, which assumes no change in the specific radioactivity of the labeled substrate, 6.7 per cent acetylation was calculated. However, since Experiments 1A and 2 were carried out concurrently and under essentially identical conditions, it may be assumed that acetylation of AF had

taken place to the same extent (38.0 per cent during 4 hours) in both experiments. Consequently, the specific radioactivity of the acetate-1-C^{14},

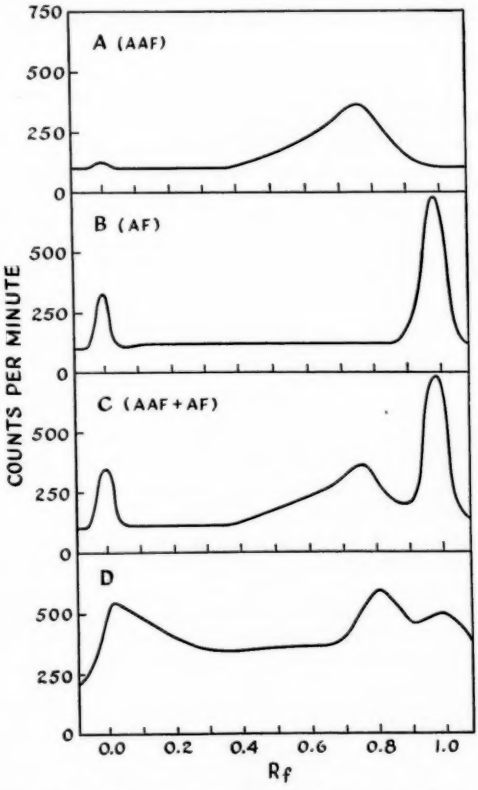

FIG. 1. Detection of AAF-9-C^{14} by ascending chromatography. The solvent system and conditions of chromatography are described in the text. The developed papers were scanned with a windowless gas flow counter (8). *A*, *B*, and *C* show the separation of pure AAF-9-C^{14} (R_F = 0.78) and AF-9-C^{14} (R_F = 0.98). *D* shows the detection of AAF-9-C^{14} (R_F = 0.83) in an extract prepared from one incubation flask of liver slices with AF-9-C^{14} as described in the text. The conditions of incubation were the same as those of Experiment 1 of Table I.

which acetylated AF, was diluted approximately 6-fold (38.0/6.7) with acetyl groups from endogenous precursors.

Complete acetylation of AF was demonstrated by the isolation of radioactive DAAF from incubation mixtures of liver slices with AF-9-C^{14} (Fig.

3). Metabolic diacetylation of either aromatic or aliphatic amines has, as far as we are aware, not been reported previously. Table II shows that 0.25 per cent and 0.16 per cent of the available AF were diacetylated in 4 hours. Replacement of the acidic hydrogen of the acetylamine by a second

TABLE I

Acetylation of 2-AF-9-C^{14} by Liver Slices

In Experiment 1, each flask contained 1.0 gm. of liver slices, 10.0 ml. of buffer, 1.9 ml. of a 4 per cent dextrose solution, and 325 γ of AF-9-C^{14} in 100 μl. of 95 per cent ethanol. In Experiment 1A, each flask contained 1.0 gm. of liver slices, 10.0 ml. of buffer, 1.8 ml. of a 4 per cent dextrose solution, 296 γ of AF-9-C^{14} in 100 μl. of 95 per cent ethanol, and 890 γ of sodium acetate in 100 μl. of distilled water. The incubation time was 4 hours; temperature, 37°; gas phase, oxygen. In both experiments the contents of three flasks were combined, and 200 mg. of carrier AAF were used.

Compound crystallized	Recrystallizing solvent	Specific activity* of recovered carrier		AAF formed		Estimated acetylation	
		Experiment 1	Experiment 1A	Experiment 1	Experiment 1A	Experiment 1	Experiment 1A
		c.p.m. per mg.	*c.p.m. per mg.*	γ	γ	*per cent*	*per cent*
AAF-9-C^{14}	50% CH_3COOH	54,500	72,600				
"	50% "	44,400	66,400				
"	50% "	44,500	64,900†				
AF-9-C^{14}	30% C_2H_5OH	44,100	67,800	285	415	23.8	38.0†

* The specific activity is expressed in counts per minute per mg. of labeled carbon 9 and is equal to (the counts per minute per mg. of the compound) × (the molecular weight of the compound)/12.

† Sample calculation,

$$\text{Mg. of metabolite} = \text{mg. of carrier} \times \frac{\text{c.p.m. per mg. C-9 of recovered carrier}}{\text{c.p.m. per mg. C-9 of substrate}}$$

$$\text{Mg. of metabolite} = 2 \times 10^3 \times \frac{6.49 \times 10^4}{3.12 \times 10^7} = 0.415 \text{ mg.} = 1.86 \ \mu\text{moles of AAF-9-C}^{14}$$

$$\left(\frac{1.86 \ \mu\text{moles AAF-9-C}^{14}}{4.9 \ \mu\text{moles substrate}}\right) \times 100 = 38.0\% \text{ acetylation}$$

acetyl group presumably takes place with greater difficulty than does monoacetylation.

To compare the extent of the acetylation reaction of AF to AAF with the deacetylation of AAF to AF, which had been demonstrated in a qualitative manner previously (2), experiments were performed in which the deacetylation reaction was measured under comparable conditions. Table III shows that, in the absence of added acetate, 23.0 per cent of AAF-9-C^{14} was hydrolyzed to AF-9-C^{14} in 4 hours. 23.8 and 19.0 per cent of AF were

acetylated to AAF under identical conditions. It would therefore appear that both reactions proceed in the liver at very similar rates. Addition of acetate to the incubation mixtures reduced the extent of deacetylation of

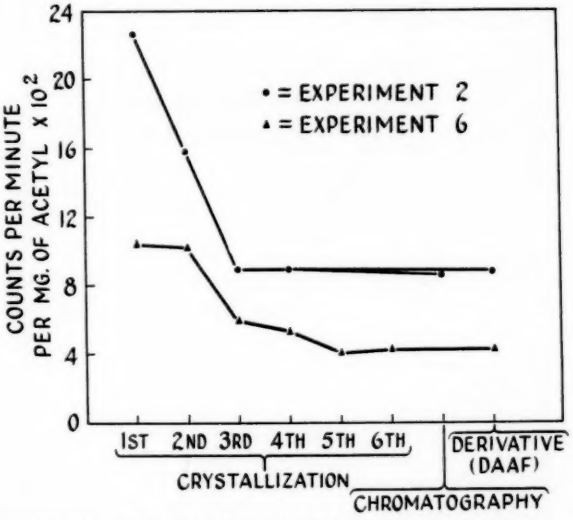

FIG. 2. Purification of acetyl-labeled AAF isolated from liver slices incubated with AF and acetate-1-C^{14} (Experiment 2) or AAF and acetate-1-C^{14} (Experiment 6). The specific radioactivity (counts per minute per mg. of acetyl) is equal to (the counts per minute per mg. of compound) × (the molecular weight of the compound)/43. Each point represents a successive purification of the isolated carrier. The first, second, fourth, and sixth crystallizations were from 50 per cent acetic acid; the third and fifth from benzene. Adsorption chromatography on alumina was carried out on a 7 × 1 cm. column with ethanol as eluent. DAAF was prepared as described in the text. The conditions of Experiment 2 were the same as in Experiment 1A, Table I, except that 300 γ of unlabeled AF in 100 μl. of 95 per cent ethanol and 870 γ of sodium acetate-1-C^{14} were the substrates. The conditions of Experiment 6 were the same as in Experiment 2, except that 300 γ of unlabeled AAF in 100 μl. of 95 per cent ethanol and 870 γ of sodium acetate-1-C^{14} were the substrates, and the incubation time was 5 hours instead of 4 hours.

AAF from 23.0 to 16.0 per cent. In contrast, the same amount of acetate increased the extent of acetylation from 23.8 to 38.0 per cent (Table I). The observation that added acetate had a quantitatively different effect raises the question whether two independent reactions rather than a reversible one are concerned with the acetylation and deacetylation of AF and AAF respectively. There is reason to believe that the acetylating and the deacetylating mechanisms are separable, since the purified arylamine aceto-

kinase of pigeon liver, which acetylates various aromatic amines through acetyl coenzyme A, had no hydrolase activity for arylacetylamines (14).

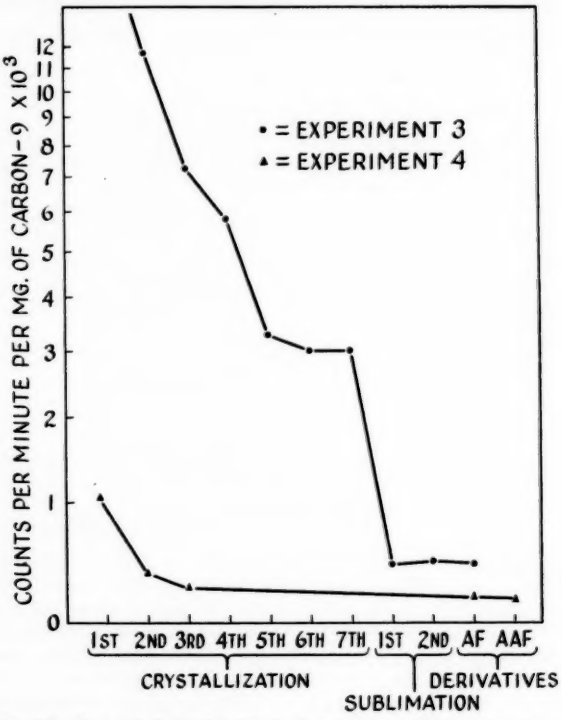

FIG. 3. Purification of DAAF-9-C^{14} isolated from liver slices incubated with AF-9-C^{14}. Each point represents a successive purification of the isolated carrier. The first, second, third, fourth, and sixth crystallizations were from 95 per cent ethanol; the fifth and seventh from methanol. The sublimations were carried out at 115° and at 0.2 mm. The derivatives were prepared as described in the text. The conditions of Experiment 3 were the same as in Experiment 1, Table I, except that 305 γ of AF-9-C^{14} were added as substrate. The contents of five flasks were combined, and 400 mg. of carrier DAAF were used. In Experiment 4 the combined mother liquors and wash liquids remaining after the crystallizations of the AAF-9-C^{14} of Experiment 1A, Table I, were filtered. The pH was adjusted to 9 with concentrated ammonium hydroxide, and the solution (65 ml.) was extracted four times with equal volumes of diethyl ether. 350 mg. of carrier DAAF were added to the ether extract, and the DAAF was reisolated.

Since both acetylation and deacetylation are carried out by rat liver slices, it appeared possible that, under physiological conditions, the re-

actions might proceed concurrently. Starting with AAF, this would result in an exchange of the acetyl groups of the carcinogen with endogenous acetyl groups. This was tested by incubation of liver slices with AAF and acetate-1-C^{14}. Fig. 4 illustrates the chromatographic detection of AAF,

TABLE II

Diacetylation of 2-AF-9-C^{14} by Liver Slices

Experiment No.*	Weight of carrier added	Final specific activity† of isolated carrier	DAAF formed	Estimated diacetylation
	mg.	*c.p.m. per mg.*	γ	*per cent*
3	400	438	5.6	0.25
4	350	182	2.0	0.16

* The experimental conditions are described in the legend to Fig. 3.
† See the foot-note to Table I.

TABLE III

Deacetylation of 2-AAF-9-C^{14} by Liver Slices

Experiment 5, the same as for Experiment 1, Table I, except that 317 γ of AAF-9-C^{14} in 100 μl. of 95 per cent ethanol were added as substrate. Experiment 5A, the same as Experiment 5. In addition, 900 γ of sodium acetate in 100 μl. of distilled water were added. In both experiments, the contents of two flasks were combined, and 200 mg. of carrier AF were used.

Compound crystallized	Recrystallizing solvent	Specific activity* of recovered carrier		AF formed		Estimated deacetylation	
		Experiment 5	Experiment 5A	Experiment 5	Experiment 5A	Experiment 5	Experiment 5A
		c.p.m. per mg.	*c.p.m. per mg.*	γ	γ	*per cent*	*per cent*
AF-9-C^{14}	Hexane	17,600	16,900				
"	"	15,500	10,600				
"	30% C_2H_5OH	15,500	10,700				
AAF-9-C^{14}	50% CH_3COOH	15,100	11,000	118	82	23.0	16.0

* See the foot-note to Table I.

which was radioactive, proving that acetyl group exchange had occurred. 2-OH-7-AAF, which had been identified previously as a metabolite of AAF in rat liver (2), was also radioactive. The data of Table IV show that replacement of the acetyl groups of either AAF or DAAF with acetate does not occur under vigorous conditions, thus excluding exchange in the absence of viable slices. Confirmation of the acetyl group exchange was obtained by the isolation of labeled AAF by the carrier method (Experiment 6, Fig. 2). Assuming for Experiment 6 the same dilution of the labeled substrate

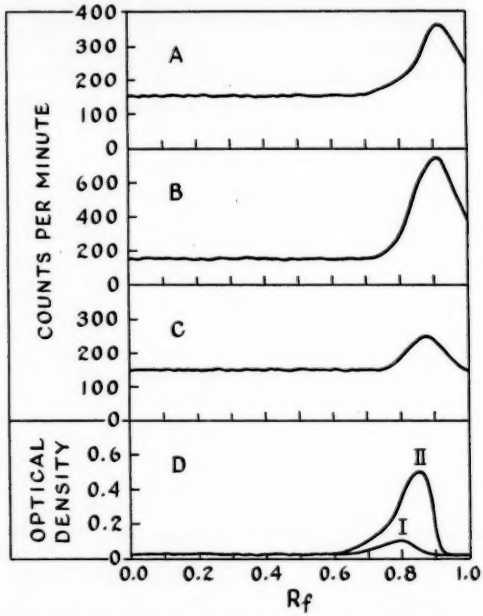

FIG. 4. Detection of acetyl-labeled AAF and 2-OH-7-AAF by ascending chromatography. Each flask contained 1.0 gm. of liver slices, 10.0 ml. of buffer, 1.8 ml. of a 1 per cent dextrose solution, 300 γ of AAF in 100 μl. of 95 per cent alcohol, and 2.18 mg. of sodium acetate-1-C^{14} in 250 μl. of distilled water. The contents of three flasks were combined, and an ethanol extract (2.0 ml.) was prepared as described in the text. Aliquots of the extract were applied to papers for chromatography. *A*, 50 μl. of the extract were subjected to electrophoresis, followed by ascending chromatography in the butanol-ammonia-water system; $R_F = 0.92$. *B*, AAF-9-C^{14} (1500 c.p.m.) was added to 50 μl. of the ethanol extract and the solution analyzed as described above; $R_F = 0.91$. *C* and *D*, 250 μl. of the ethanol extract were applied to the paper, and the radioactive AAF was removed by migration in the hexane-benzene-water system as described in the text. *C* shows a radioactive peak after the cut paper had been developed in the butanol-ammonia-water system; $R_F = 0.87$. *D-I* is a plot of optical densities *versus* R_F after spraying with diazotized sulfanilic acid; $R_F = 0.80$. In *D-II*, 9 γ of 2-OH-7-AAF were added to another aliquot in order to enhance the color peak ($R_F = 0.86$), thus demonstrating the identity of the radioactive peak *C* with 2-OH-7-AAF.

(5.7-fold) by endogenous acetyl groups as that indicated by Experiments 1A and 2, which were carried out under essentially identical conditions, it may be calculated[3] that 22.4 per cent of the acetyl groups of AAF had been

[3] Calculation of exchange: (2.42×10^6 c.p.m. per mg. of acetyl group of the substrate)/5.7 = 4.25×10^5 c.p.m. per mg. of acetyl group after dilution. Specific ac-

replaced by endogenous acetyl groups. On the basis of the measurements of the individual reactions (16 per cent deacetylation and 38 per cent acetylation) in the presence of acetate, one obtains a maximal calculated value of 16 × 0.38 or 6.1 per cent for the exchange. The greater extent of

TABLE IV

Absence of Chemical Exchange of Acetyl Group of AAF and DAAF with Acetate-1-C¹⁴

Compound*	Crystallizing solvent	Specific activity	
		Crystallization 1	Crystallization 2
		c.p.m. per mg.	*c.p.m. per mg.*
AAF	50% CH_3COOH	3	0
DAAF	95% C_2H_5OH	0	

* To 50 mg. of the compounds in the solvents were added 87 γ of sodium acetate-1-C¹⁴ (10^5 c.p.m.). After refluxing the solutions for ½ hour, the compounds were isolated.

TABLE V

Protein Binding of Radioactivity by Rat Liver

In Experiment 7, each flask contained 1.0 gm. of liver slices, 10.0 ml. of buffer, and 1.8 ml. of a 4 per cent dextrose solution. In addition, the flask in Experiment 7A contained 153 γ of AF-9-C¹⁴; in Experiment 7B, 305 γ of AF-9-C¹⁴; and in Experiment 7C, 610 γ of AF-9-C¹⁴ in 200 μl. of 95 per cent ethanol. Other conditions were the same as described in Table I.

Experiment No.	Substrate		Specific activity of tissue after extraction* with			
	Compound	Concentration	C_2H_5OH	C_6H_6	$(C_2H_5)_2O$†	C_2H_5OH
		γ per ml.	*c.p.m. per mg.*	*c.p.m. per mg.*	*c.p.m. per mg.*	*c.p.m. per mg.*
7A	AF-9-C¹⁴	13	52	46	47	43
7B	"	25	75	76	71	69
7C	"	51	141	142	143	136

* The extractions were performed with boiling solvents for 18 to 24 hours.

† After this extraction 50 mg. of the tissue were dissolved in 5 ml. of 10 per cent sodium hydroxide solution. The tissue was precipitated with 6 ml. of cold 50 per cent trichloroacetic acid solution and reextracted with ethanol.

replacement in Experiment 6 suggests that the acetyl group exchange is not adequately described by one reversible reaction. The experimental evidence favors the view that deacetylation of AAF to AF and reacetylation

tivity of the isolated AAF = 430 c.p.m. per mg. of acetyl group. Carrier AAF added = 200 mg. Mg. of AAF formed by exchange = $(200 \times 430)/(4.25 \times 10^5)$ = 0.202 mg. = 0.906 μmole. Total substrate AAF = 4.04 μmoles; $(0.906/4.04) \times 100$ = 22.4 per cent exchange.

of AF occur repeatedly in a cyclic fashion. It follows that the values measured for the single reactions over a 4 hour period (Tables I and III) would represent net values resulting from the operation of the cycle.

It had previously been shown that the radioactivity of AAF-9-C^{14} was chemically bound to liver slice proteins (2). In so far as slices hydrolyzed AAF-9-C^{14} to AF-9-C^{14}, it was conceivable that the bound radioactivity was contributed by AF-9-C^{14}. Employing the criteria for protein binding which had previously been defined, we observed chemical binding of the radioactivity of AF-9-C^{14} (Table V). The extent of binding was a function of the concentration of the labeled substrate, as in the case of AAF-9-C^{14} (2). In view of the metabolic interchange of the two compounds, further experiments are required to determine which compound or compounds were bound to proteins.

DISCUSSION

The present experiments provide experimental evidence that the liver accomplishes the acetylation of AF. It is therefore likely that a major portion, if not all, of the conjugated amino groups observed spectrophotometrically after injection of the carcinogen (1) is due to acetylated products. It has also been shown that acetylation and deacetylation take place simultaneously within the liver. It follows that AF and AAF must always occur together regardless of which compound is fed. The metabolic interchangeability of AF and AAF demonstrated here would seem to defy attempts to designate either compound as primarily responsible for the initiation of carcinogenesis (15, 16). The precise definition of the *primary* carcinogen is further complicated by the isolation of DAAF as a metabolite of AF (and therefore presumably of AAF), inasmuch as DAAF and AAF have equal carcinogenic potency (5).

The demonstration of the acetylation of AF by rat liver slices raises the question whether the carcinogen interacts with acetyl coenzyme A and an acetokinase in a manner similar to sulfanilamide and other arylamines (17, 18). The acetyl exchange can be accounted for by deacetylation of AAF, possibly through a specific deacetylase for arylacylamines (19), which is followed by reacetylation of the AF formed by acetyl coenzyme A and an acetokinase. It appears pertinent in this regard that coenzyme A is especially abundant in rat liver (17). Support for this mode of acetylation of AF may be derived from the finding of Allison *et al.* that the urinary excretion of ether-soluble, conjugated material (identified as AAF by ultraviolet spectroscopy) by dogs fed AF may be increased by a high intake of pantothenate (20). On the other hand, the absence of acetylsulfanilamide in dog urine after administration of sulfanilamide (21) raises some doubt as to whether AF and sulfanilamide are necessarily acetylated by the same

mechanism. Should future experiments establish the involvement of acetyl coenzyme A and an acetokinase, then both AF and AAF could function to trap high energy acetyl groups and the prolonged administration of AAF might divert compounds containing high energy bonds from physiological pathways. In view of the apparent cyclic nature of the deacetylation and reacetylation, this effect could be of considerable magnitude and might conceivably interfere with normal metabolism.

SUMMARY

1. Acetylation of 2-aminofluorene to 2-acetylaminofluorene and deacetylation of 2-acetylaminofluorene to 2-aminofluorene have been shown to take place in rat liver slices. The stimulation of the acetylation of AF by unlabeled acetate and isolation of acetyl-labeled AAF after incubation of slices with AF and acetate-1-C^{14} indicate that acetate (presumably in the form of acetyl coenzyme A) may serve as the acetyl group donor. The magnitudes of the acetylation and the deacetylation reaction have been compared by means of isotope carrier techniques. The effect of added acetate on the two reactions suggests separate reaction mechanisms rather than a reversible reaction.

2. 2-Diacetylaminofluorene was synthesized in small amounts from 2-aminofluorene by rat liver slices.

3. The simultaneous occurrence of the deacetylation of 2-acetylaminofluorene and the acetylation of 2-aminofluorene in rat liver slices has been demonstrated.

4. Chemical binding of the radioactivity to rat liver proteins has been found after incubation of slices with 2-aminofluorene-9-C^{14}. Extent of binding and concentration of the carcinogen were proportional.

The authors wish to thank Mrs. Dorothy Filbin for assistance with the carbon combustions.

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THE ENZYMATIC CLEAVAGE OF β-HYDROXY-β-METHYLGLUTARYL COENZYME A TO ACETOACETATE AND ACETYL COENZYME A*

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It is known from previous isotopic studies (2–6) that the formation of acetoacetate from isovalerate (an intermediate in leucine metabolism) involves two chemical changes which are of particular interest because they do not occur in the course of straight chain fatty acid metabolism. These reactions are carbon dioxide fixation and cleavage of the carbon chain without the prior formation of a β-keto acid. It was suggested as a working hypothesis that cleavage of the isopropyl group from isovalerate might occur prior to the carboxylation step (5). Attempts in this laboratory to obtain evidence for the postulated 3-carbon intermediate were unsuccessful, however, and more recent enzymatic studies have led to the proposal of the following reaction sequence in which carboxylation occurs prior to the cleavage reaction (7, 8).

(1) Isovaleryl CoA $\rightleftharpoons$ β-methylcrotonyl CoA[1] (+2H)

(2) β-Methylcrotonyl CoA + H_2O $\rightleftharpoons$ β-hydroxyisovaleryl CoA

(3) β-Hydroxyisovaleryl CoA + CO_2 $\xrightleftharpoons{\text{ATP}}$ β-hydroxy-β-methylglutaryl CoA

(4) β-Hydroxy-β-methylglutaryl CoA $\rightleftharpoons$ acetoacetate + acetyl CoA

The oxidation of isovaleryl CoA to the α,β-unsaturated thiol ester (Reaction 1) is analogous to the corresponding reaction with straight chain fatty acids (9, 10). The hydration of β-methylcrotonyl CoA (Reaction 2)

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[1] The following abbreviations are used: reduced coenzyme A, CoA; thiol ester of coenzyme A, acyl CoA; β-hydroxy-β-methylglutaric acid, HMG; mono CoA ester of β-hydroxy-β-methylglutaric acid, HMG CoA; adenosine triphosphate, ATP; tris-(hydroxymethyl)aminomethane, Tris.

is catalyzed by crotonase, which is present in both heart and liver extracts (11, 12). Heart extracts which have been freed of crotonase convert β-hydroxyisovaleryl CoA (but not β-methylcrotonyl CoA) to acetoacetate and acetyl CoA when supplemented with ATP and bicarbonate. The postulated intermediate, HMG CoA, has been synthesized and has been shown to undergo rapid enzymatic cleavage (Reaction 4) to give the expected products in almost equimolar amounts. The present paper is concerned chiefly with the purification and properties of the HMG CoA cleavage enzyme.

EXPERIMENTAL

Enzyme Assay—The assay which was employed is based on the enzymatic cleavage of HMG CoA, followed by deproteinization and estimation of the acetoacetate formed. The following were added to a tube chilled in ice: Tris buffer, pH 8.1, 200 μmoles; L-cysteine, 13 μmoles; magnesium chloride, 20 μmoles; HMG CoA, 2 μmoles. To this mixture the enzyme solution (diluted when necessary in 0.1 M Tris buffer, p.H 7.4, containing 0.2 mg. of crystalline egg albumin per ml.) was added and the final volume was adjusted to 1.0 ml. with water. The tube was flushed with nitrogen, stoppered, and incubated in a water bath at 37° for 60 minutes. At the end of this time the tube was placed in ice and 0.35 ml. of 40 per cent trichloroacetic acid was added. The mixture was centrifuged, and an aliquot of the supernatant solution was used for the determination of acetoacetate by a modification of the method of Barkulis and Lehninger (13). Controls without added enzyme and without added substrate were included and the amount of acetoacetate found in them was subtracted from that found in the other tubes.[2] The rate of acetoacetate synthesis was found to be dependent on the concentration of the cleavage enzyme over a limited range. This range can be extended by employing higher concentrations of HMG CoA, but the cost of using larger amounts of CoA for this purpose is prohibitive at the present time. 1 unit of cleavage enzyme is defined as that amount which will catalyze the formation of 1.0 μmole of acetoacetate under the conditions described. The specific activity of the enzyme is expressed as the number of units per mg. of protein. The protein concentration of the enzyme solutions was determined spectrophotometrically by light absorption at 280 and 260 mμ, with a correction for the nucleic acid content (14).

Extraction and Purification of Enzyme—The steps employed in purifying the cleavage enzyme 17-fold from heart extracts are outlined in Table I.

[2] Some commercial preparations of coenzyme A contain traces of a substance which is detected by the color reaction employed. As indicated by paper chromatography, this impurity is probably acetone.

Unless otherwise stated, the operations were carried out at 0°. Pig hearts obtained promptly after death of the animals were packed in ice, trimmed of fat and connective tissue, and ground in a mechanical mincer. 500 gm. of the minced tissue were homogenized with 500 ml. of 0.5 M potassium chloride in a Waring blendor for about 3 minutes. The resulting thick suspension was diluted with 500 ml. of 0.5 M potassium chloride; 10 ml. of 1 M potassium phosphate buffer, pH 7.4, were added and the preparation was stirred mechanically for 30 minutes. It was then placed in a room at −20° and 1 liter of alcohol solution (prepared by mixing equal volumes of absolute ethanol and 0.5 M potassium chloride), previously chilled to −20°, was added slowly with stirring during 1 hour. The resulting mixture, containing 20 volumes per cent alcohol, now at a temperature of about −7°, was centrifuged at 800 × *g* for 2 hours at −5°. The clear, red supernatant solution was dialyzed for 16 hours against 40 liters of potassium chloride, 0.04 M, containing potassium phosphate buffer, pH 7.4, 0.025 M, and L-cysteine, 0.001 M. The dialyzed extract contained 7.5 gm. of protein in a volume of 1.7 liters.

TABLE I

Partial Purification of Cleavage Enzyme from Pig Heart

Preparation	Protein	Units	Specific activity	Yield
	mg.		*units per mg.*	*per cent*
Extract	7500	3200	0.4	100
Ammonium sulfate ppt. (0–0.7 saturation)	3000	2500	0.8	78
" " " (0–0.55 ")	1240	1890	1.5	59
Isoelectric ppt	360	960	2.7	30
Ammonium sulfate ppt. (0.13–0.32 saturation)	97	680	7.0	21

The extract was brought to a final ammonium sulfate concentration of 0.37 gm. per ml. (0.7 saturation) by the slow addition of 45.4 gm. of the salt per 100 ml. of extract. The mixture was stirred for 30 minutes and centrifuged for 20 minutes at 13,000 r.p.m. The precipitate was dissolved in a minimal volume of 0.1 M Tris buffer, pH 7.4, and dialyzed overnight against 3 liters of the same buffer containing 0.001 M cysteine. The resulting pink solution contained 3.0 gm. of protein in a volume of 150 ml.

The preparation was then brought to an ammonium sulfate concentration of 0.29 gm. per ml. (0.55 saturation) by the addition of 50.8 gm. of salt. The precipitate was collected and dissolved as in the previous step and dialyzed for 36 hours against 700 ml. of 0.06 M Tris, pH 7.4, containing 0.001 M cysteine, with replacement of the buffer-cysteine solution at 18

hours. This preparation contained 1.24 gm. of protein in a volume of 35 ml.

The solution was diluted with an equal volume of distilled water, and 1 M acetic acid was added dropwise with efficient stirring until the pH fell to 5.8. The mixture was stirred for 15 minutes, allowed to stand for 1 hour, and centrifuged at 20,000 × *g* for 20 minutes. The precipitate was brought into solution in 30 ml. of 0.1 M Tris buffer, pH 7.4, containing 0.001 M cysteine. The resulting solution contained 360 mg. of protein.

The enzyme solution was brought to an ammonium sulfate concentration of 0.07 gm. per ml. (0.13 saturation) by the addition of 4.6 ml. of a saturated solution of the salt. The precipitate obtained upon centrifugation was discarded, and the supernatant solution was brought to a salt concentration of 0.17 gm. per ml. (0.32 saturation) by the addition of 9.7 ml. of saturated ammonium sulfate solution. The precipitate obtained upon centrifugation was dissolved in 10 ml. of 0.1 M Tris buffer, pH 7.4, containing 0.001 M cysteine. The resulting clear, colorless solution contained 97 mg. of protein. This preparation was found to be stable to storage in the frozen state at −20°.

TABLE II

Distribution of Cleavage Enzyme

Tissue extracted	Specific activity
	units per mg.
Pigeon liver	1.8
Pig kidney	1.0
" heart	0.9
" liver	0.7
Rat brain	0

The specific activity found for pig heart extract in this experiment was somewhat higher than the usual value (see Table I). The three pig tissues indicated were obtained on the same day and extracted and assayed at the same time.

Distribution of Enzyme—The various tissues listed in Table II were extracted with potassium chloride in 20 per cent ethanol, dialyzed, and assayed as described above. The cleavage enzyme was found to be present in about the same concentration in extracts of pig liver, kidney, and heart, but could not be detected in an extract of rat brain. Pigeon liver appears to have about twice the cleavage activity of pig heart. In general, aqueous buffer extracts and acetone powder extracts of tissues were found to contain less of the cleavage enzyme than the alcohol extracts described.

Optimal pH—The cleavage enzyme is active over the pH range 7.4 to 10.5, with maximal activity at about 10.0 (Fig. 1). The conversion of

acetoacetate to the enol form or the destruction of acetyl CoA at alkaline pH may enhance the apparent activity of the cleavage enzyme by removal of the products of the reaction. The experimental evidence available at the present time, however, is insufficient for evaluating this possibility.

Thiol and Divalent Metal Requirement—The cleavage enzyme has no appreciable activity in the absence of an added thiol. Cysteine is used routinely for this purpose, but several other thiols are equally or somewhat more effective (Table III). Thiomalic acid may act as an inhibitor for the cleavage reaction, for malic acid has been found to have this effect. In several instances in which magnesium ion was omitted from the assay system the cleavage enzyme had diminished activity. Definite evidence for this metal requirement has been obtained by experiments carried out in the presence of ethylenediaminetetraacetic acid (Versene), as indicated in Table IV. In the presence of this chelating agent the enzyme exhibits no measurable activity unless supplemented with magnesium ions. Manganese can be substituted for magnesium, but cobalt is ineffective at the concentration employed.

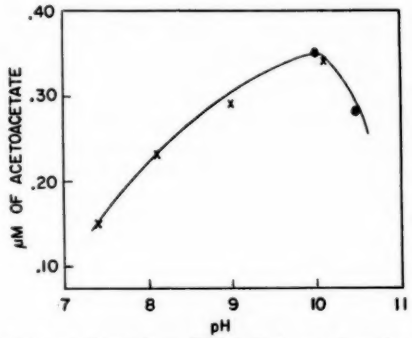


FIG. 1. Acetoacetate synthesis from HMG CoA as a function of pH. The assay described in the text was employed, with 0.23 mg. of cleavage enzyme of specific activity 7.0 in all cases, 400 μmoles of Tris buffer and 2 μmoles of HMG CoA as indicated by ×, and 200 μmoles of glycine buffer and 3 μmoles of HMG CoA as indicated by ●.

Substrate Specificity—The expense of using stoichiometric amounts of HMG CoA in the assay employed has hindered the further purification of the cleavage enzyme. In an attempt to circumvent this limitation, certain other compounds have been tested as possible substrates for the cleavage enzyme. HMG (free acid) and β-hydroxyisovaleryl CoA do not serve as substrates, as tested by acetoacetate formation, nor do the HMG thiol esters of glutathione, cysteine, or *N*-acetylthioethanolamine, even when

tested at elevated levels. The failure of β-hydroxyisovaleryl CoA to undergo cleavage to furnish acetone and acetyl CoA is in complete accord

TABLE III

Thiol Requirement for Cleavage Activity

Experiment No.	Thiol added	Acetoacetate formed (per cent of yield with cysteine added)
1	Cysteine	100
	Glutathione	113
	None	9
2	Cysteine	100
	Thioglycolate	136
	Glutathione	145
	Thioethanolamine	127
	Thiomalate	23

The usual test system containing 1 μmole of HMG CoA as substrate was employed. In Experiment 1, a fraction of pig heart precipitated by 32 to 50 per cent ethanol served as a source of the cleavage enzyme (0.49 mg. of protein per tube). In Experiment 2, the enzyme used was the 0–0.55 ammonium sulfate fraction referred to in Table I (2.86 mg. of protein per tube). The thiols were neutralized with dilute potassium hydroxide immediately before they were added to the assay system (13 μmoles per tube).

TABLE IV

Metal Requirement for Cleavage Activity

Experiment No.	Metal added	Versene added	Acetoacetate formed
		μmoles	*μmole*
1	Mg^{++}	0	0.18
	"	10	0.17
	None	10	0.02
2	Mg^{++}	20	0.19
	Mn^{++}	20	0.13
	Co^{++}	20	0.03

The usual test system containing 2 μmoles of HMG CoA as substrate was employed. In Experiment 1, 0.24 mg. of the 0.13–0.32 ammonium sulfate fraction of heart was added to each tube and 50 μmoles of $MgCl_2$ as indicated. In Experiment 2, 0.60 mg. of the enzyme was added to each tube and 100 μmoles of $MgCl_2$, $MnSO_4$, or $CoCl_2$ as indicated. In all instances, the Versene was adjusted to pH 8.1 with potassium hydroxide before it was added to the tubes.

with the earlier demonstration that acetoacetate, not acetone, is the metabolic end-product of the isopropyl group of leucine (5).

Identification of Products of Cleavage Reaction—In an earlier brief communication (7), an experiment is referred to in which HMG CoA was in-

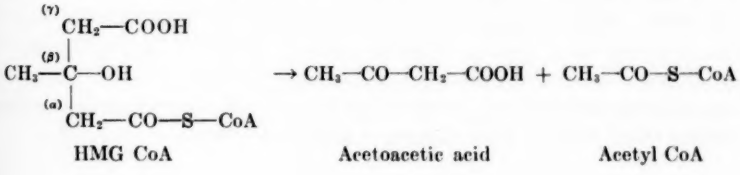
cubated with cleavage enzyme, the reaction mixture was deproteinized, and aliquots were taken for the independent determination of acetoacetate (13) and acetyl CoA, the latter by citrate formation in the presence of oxalacetate and crystalline citrate condensing enzyme. The molar ratio of acetyl CoA to acetoacetate formed was found to be 0.75. Under the conditions employed prior to the deproteinization step (incubation for 1 hour at 38°, pH 8.1), about 26 per cent of a sample of known acetyl CoA was found to be destroyed, as assayed by citrate formation. It is clearly evident, therefore, that acetoacetate and acetyl CoA must be formed in equimolar amounts in the enzymatic splitting of HMG CoA.

Additional proof for the identity of the cleavage products has been obtained with the aid of paper chromatographic methods. After incubation of 2 μmoles of HMG CoA with heart cleavage enzyme in the presence of magnesium chloride and cysteine, the thiol esters present were converted to the hydroxamic acids (15) and submitted to paper chromatography in water-saturated phenol. The product (R_F 0.58) was identified by comparison with the values obtained for known acetohydroxamic acid (R_F 0.55) and HMG hydroxamic acid (R_F 0.17) under identical conditions. As expected, in a control experiment in which the substrate was omitted, no hydroxamic acids were detected.

For identification of acetoacetate as the other product of HMG CoA cleavage, 5 μmoles of the latter compound were incubated with heart cleavage enzyme (specific activity 7.0) under the usual assay conditions, and 2,4-dinitrophenylhydrazine was then added to trap carbonyl compounds. The mixture was extracted according to established methods (16) and submitted to paper chromatography in 95 per cent methanol. A single yellow spot (R_F 0.46) was obtained, corresponding exactly to the R_F of known acetoacetate dinitrophenylhydrazone and clearly distinguishable from acetone dinitrophenylhydrazone (R_F 0.78). In a control experiment without added substrate, the only substance detected was a trace of free dinitrophenylhydrazine (R_F 0.57).

DISCUSSION

From the evidence presented it is apparent that the cleavage of HMG CoA occurs between the α- and β-carbons as illustrated. If the cleavage



were to occur between the β- and γ-carbons, acetate and acetoacetyl CoA would be the products expected. These products could not account for the results obtained, however, since acetoacetyl CoA is measurable as acetoacetate only to the extent of about 10 per cent under the analytical conditions employed (17). Furthermore, acetoacetyl CoA could not have been converted to acetoacetate enzymatically in these experiments, for this reaction occurs in dialyzed heart preparations only upon the addition of succinate to activate the CoA transferase reaction (18, 19). It should also be pointed out that acetoacetyl CoA, if formed, would promptly give rise to acetyl CoA because of the presence of thiolase (18, 20, 21) in the alcohol-potassium chloride extracts of heart. The formation of acetyl CoA in this manner would, of course, be incompatible with the observation that HMG CoA produces equimolar amounts of acetoacetate and acetyl CoA. These conclusions concerning the asymmetric cleavage of HMG CoA are in agreement with earlier isotopic studies on the conversion of C^{14}-labeled leucine and isovalerate to acetoacetate and acetyl CoA in liver tissue.

The cleavage reaction under discussion is obviously similar in nature to the reversible formation of citrate from oxalacetate and acetyl CoA, catalyzed by the citrate condensing enzyme described by Stern and Ochoa (22). It was readily demonstrated that the enzymes are not identical, however, for a crystalline preparation of the citrate condensing enzyme had no detectable activity in the HMG CoA cleavage assay. A different enzyme involved in the cleavage of citrate to oxalacetate and acetyl CoA in the presence of ATP, CoA, and magnesium ions has recently been reported by Srere and Lipmann (23). They were unable to obtain evidence for citryl CoA as an intermediate product, and the relationship of their enzyme to the HMG CoA cleavage enzyme is uncertain at the present time.

Investigators in the field of steroid biosynthesis (notably Gurin and Bloch and their associates and Rudney) have provided evidence for the biosynthesis of labeled HMG from C^{14}-labeled acetate in liver preparations and are currently studying the possible rôle of HMG and related branched chain acids in squalene and cholesterol formation (24–28). These workers are investigating HMG formation by an acetoacetate-acetate condensation which would be similar to the reversal of HMG CoA cleavage. Preliminary studies indicate that the HMG CoA cleavage reaction is not readily reversible, thereby suggesting that a somewhat different reaction may be the primary step leading to intermediates in cholesterol synthesis. If products of leucine degradation are directly involved in steroid formation, this will represent the only known major metabolic rôle of leucine in animal tissues other than its participation in protein synthesis.

Methods

HMG was synthesized by ozonization of diallylmethylcarbinol according to the procedure of Klosterman and Smith (29) and recrystallized from anhydrous ether solution by the addition of heptane. Thiol esters of HMG were prepared by the method of Wieland and Rueff (30), with equimolar amounts of HMG, pyridine, and ethyl chloroformate to insure formation of the monoanhydride and in turn the monothiol ester. The concentration of thiol esters made in this manner was estimated on the basis of thiol sulfhydryl disappearance, as determined by the method of Grunert and Phillips (31). When samples of the synthetically prepared HMG CoA were chromatographed in ethanol-acetate (32) on Whatman No. 3 paper, the expected thiol ester spot was obtained (R_F 0.59), but no other thiol ester or free sulfhydryl compounds were detectable.

CoA and crystalline egg albumin were commercial products. Crystalline citrate condensing enzyme was kindly furnished by Dr. J. R. Stern and Dr. S. Ochoa. Dr. H. J. Klosterman generously furnished a sample of HMG and the details of his preparation of the compound prior to publication.

The authors wish to acknowledge the technical assistance of Miss Theresa I. Jendrejewski.

SUMMARY

1. An enzyme which catalyzes the cleavage of HMG CoA to equimolar amounts of acetoacetate and acetyl CoA was demonstrated to be present in extracts of liver, kidney, and heart. The enzyme was purified about 15-fold from extracts of pig heart by ammonium sulfate fractionation and isoelectric precipitation. The enzyme assay was based on acetoacetate formation from synthetically prepared HMG CoA.

2. The cleavage reaction requires the presence of Mg^{++} or Mn^{++} ions and a thiol compound such as cysteine, glutathione, thioethanolamine, or thioglycolate. The enzyme is active over the pH range 7.4 to 10.5, with maximal activity at about pH 10.0.

3. HMG and its thiol esters of glutathione, cysteine, or *N*-acetylthioethanolamine are not attacked by the enzyme, nor is β-hydroxyisovaleryl CoA.

4. The results obtained are in agreement with the conclusion reached from earlier isotopic studies that acetoacetate, not acetone, is the metabolic end-product of the isopropyl group of leucine.

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ENZYMATIC REDUCTION OF PYRIDINE NUCLEOTIDES BY MOLECULAR HYDROGEN*

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Since the original description of hydrogenase by Stephenson and Stickland (1), several workers have provided evidence indicating that various microorganisms are capable of catalyzing the reduction of oxidation-reduction dyes and metabolic intermediates by molecular hydrogen. The demonstration by Stadtman and Barker (2) that cell-free extracts of *Clostridium kluyveri* catalyze the reduction of acetaldehyde by molecular hydrogen strongly suggests the participation of pyridine nucleotides as intermediate hydrogen acceptors in this system. The present paper provides evidence that the reduction of both diphosphopyridine nucleotide and triphosphopyridine nucleotide by molecular hydrogen is catalyzed by cell-free preparations of *C. kluyveri* and that heat-stable cofactors are required for this reaction.

A preliminary report of these findings has appeared elsewhere (3). A recent note by Peck and Gest (4) presents confirmatory evidence for the observations reported here.

EXPERIMENTAL

Methods and Materials

DPN, CoA, TPN, and glutathione were obtained commercially.[1] Potassium pyruvate and lactic dehydrogenase were prepared as described previously (5).

The cofactor solution referred to was prepared by additions of 1 gm. of dried cells to 20 ml. of boiling water and maintenance of the temperature for 10 minutes, followed by centrifugation. The residue was reextracted with 10 ml. of boiling water as before, and the supernatant solution was combined with the first extract after centrifugation. The lipoic acid was a gift from the Lederle Laboratories. The lipothiamide pyrophosphate

* This project was initiated at the National Heart Institute, National Institutes of Health, Bethesda, Maryland, while the author was on leave of absence from the Department of Pharmacology, New York University College of Medicine, New York.

[1] The following abbreviations are used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; GSSG and GSH, oxidized and reduced glutathione; CoA, coenzyme A; Tris, tris(hydroxymethyl)aminomethane.

was generously supplied by Dr. P. K. Stumpf. Much of the work reported here was performed with a preparation of *C. kluyveri* grown by Dr. E. R. Stadtman, in whose laboratory this project was initiated.

Sulfhydryl determinations were performed on metaphosphoric acid filtrates by a modification of the method of Grunert and Phillips (6), in that saturated ammonium sulfate was employed in place of sodium chloride and colorimeter readings were made 30 seconds after the last addition. In later experiments, 1.5 M K_3PO_4 was substituted for sodium carbonate in this procedure, which eliminated the occasionally troublesome formation of gas bubbles. Pyruvate was determined on perchloric acid-precipitated samples by the method of Friedemann and Haugen (7).

Preparation of Enzyme—C. kluyveri was grown in 50 gallon stainless steel drums, harvested by centrifugation in a Sharples ultracentrifuge, and the resulting cell paste dried in a vacuum desiccator, according to the procedure of Stadtman and Barker (8). The dried cells were stored at −15°.

10 gm. of dried cells were ground in a mortar to a powder and added to an Erlenmeyer flask containing 150 ml. of a solution of 0.01 M potassium phosphate and 0.01 M cysteine, pH 7.0, which had previously been deaerated by evacuation and flushing with hydrogen. The flask was flushed with H_2, stoppered, and agitated for 4 hours at room temperature by means of a magnetic stirrer. After centrifugation at 15,000 × *g* for 20 minutes, the clear, yellow[2] supernatant fluid was transferred to a container which was alternately evacuated and refilled with hydrogen. The residue from centrifugation was reextracted with 75 ml. of phosphate-cysteine solution, and the supernatant fluid after recentrifugation was combined with the initial extract. This may be stored at 0° for 2 weeks without appreciable loss of activity; when stored at −15°, it is stable for at least 2 months.

Dialysis of this preparation was carried out against 40 to 50 volumes of 0.01 M potassium phosphate-0.005 M cysteine buffer, pH 7, under H_2, for varying periods, as indicated below.

Properties of Enzyme

DPN-Mediated Reduction of Pyruvate—If an extract dialyzed 3 hours is incubated in a Warburg vessel gassed with hydrogen, gas consumption is observed for the first 40 minutes, at which point it is essentially complete. If, after the endogenous reaction has apparently ceased, a mixture of pyruvate, DPN, and lactic dehydrogenase is added from a side arm, gas uptake resumes at a markedly increased rate (Fig. 1). Omission of either pyruvate or lactic dehydrogenase results in only slight activity; omission of DPN

[2] The color of the extracts is a good index of anaerobiosis. The completely reduced preparation is a light amber. On exposure to air the preparation darkens considerably and may show a greenish hue.

results in a rate which is one-half the rate when all components are present. With limiting quantities (5 μmoles) of pyruvate, hydrogen consumption ceases when approximately 1 equivalent of gas has been taken up, in

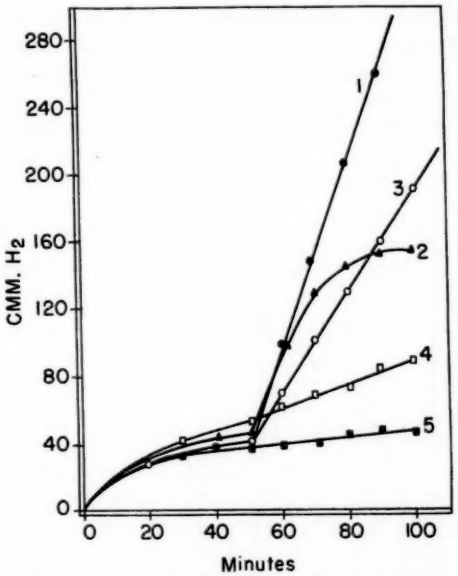


FIG. 1. Reduction of pyruvate by hydrogen. Curve 1, the main space of the Warburg vessel contained 200 μmoles of Tris buffer, pH 8.0, 6 μmoles of cysteine, and 0.5 ml. of extract dialyzed 3 hours. After 50 minutes, a mixture containing 50 μmoles of potassium pyruvate, 0.15 μmole of DPN, and 5×10^3 units of lactic dehydrogenase was tipped from the side bulb. Gas, H_2; temperature, 30°; final volume, 2.0 ml. Curve 2, 5 μmoles of potassium substituted for the 50 μmoles of Curve 1. Curve 3, DPN omitted; Curve 4, lactic dehydrogenase omitted; Curve 5, pyruvate omitted.

accordance with the over-all equation

(1) $$H_2 + \text{pyruvate} \rightleftharpoons \text{lactate}$$

Reduction of DPN by Hydrogen—Initial experiments designed to demonstrate spectrophotometric reduction of DPN by gaseous hydrogen yielded equivocal results. On examination two sources of difficulty were revealed. The first was the presence of a powerful DPNH oxidase in the extract, of which the activity is considerably greater than that of hydrogenase. As a result, traces of oxygen introduced in the course of making additions to the spectrophotometer cuvettes reversed any reduction of DPN that may have occurred. Secondly, these extracts manifest broad absorption from

the near ultraviolet well into the visible region. A large contribution to this absorption seems to be made by flavins, since several components with characteristic flavin spectra may be separated by paper chromatography from extracts deproteinized by boiling. Some color may be due to residual traces of methylene blue, which is employed as an indicator for anaerobiosis during the washing of the harvested organisms. All of the color in the extract is bleached after a short incubation under hydrogen and considerably more rapidly in the presence of DPN. The result, in the presence of trace amounts of DPN, is a *decrease* in absorption at 340 mμ as well as in

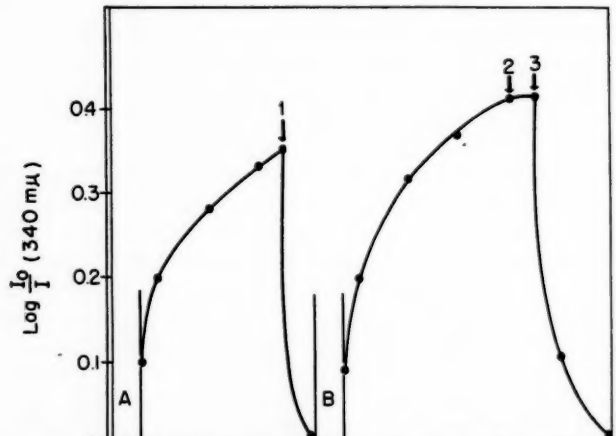


FIG. 2. Reduction of DPN by hydrogen. Zones A and B represent periods of gassing with hydrogen. For other conditions, see the text.

the visible region upon equilibration with hydrogen. It was subsequently observed that a number of reducing agents, notably sulfhydryl compounds, caused a marked decrease of absorption in the enzyme preparation in an atmosphere of helium or nitrogen. The experiment indicated in Fig. 2 was performed by taking advantage of this property. A mixture containing 0.6 ml. of undialyzed extract, 600 μmoles of Tris buffer, pH 8.0, 30 μmoles of cysteine, and 1.0 μmole of DPN in a final volume of 6.0 ml. was gassed with helium in a wide bore test-tube for 5 minutes. Two equal aliquots of 2.8 ml. were transferred to Beckman cuvettes under a stream of helium, the cuvettes were covered, and changes in optical density were observed at 340 mμ against a water blank. In both cuvettes, the absorption steadily decreased over the course of 15 minutes, from which time the values

were constant and equal. At this time, the contents of one of the cuvettes were transferred to a wide bore test-tube and agitated by a stream of hydrogen for 3 minutes, then returned to the cuvette which again was covered. The initial portion of Fig. 2 represents the change in optical density with the time relative to the aliquot under helium. At Arrow 1, the contents of the experimental cuvette were again transferred to a wide tube, agitated in air for 1 minute, then poured back into the cuvette. As is seen in the graph, the 340 mμ absorption increment was completely abolished by this treatment. The effect is attributable to the DPNH oxidase previously referred to. Upon repeating the gassing with hydrogen, the 340 mμ absorption again increased with time. The addition of lactic dehydrogenase (2000 units, Arrow 2), followed by 5 μmoles of pyruvate (Arrow 3), resulted in an extremely rapid decline in absorption, adding further weight to the conclusion that reduced DPN was the substance responsible for the increment in absorption. Taken together with the data on the reduction of pyruvate, the conclusion that Reaction 1 is the sum of the following two reactions seems warranted.

$$H_2 + DPN^+ \rightleftharpoons DPNH + H^+ \tag{2}$$

$$DPNH + H^+ + \text{pyruvate} \rightleftharpoons \text{lactate} + DPN^+ \tag{3}$$

TPN-Mediated Reduction of Oxidized Glutathione—If an extract dialyzed 3 hours is incubated in a Warburg vessel under hydrogen until endogenous gas consumption has essentially ceased and a mixture of oxidized glutathione, TPN, and glutathione reductase[2] is then added, a further uptake of hydrogen is observed (Fig. 3). The extent of dependence on the various additions is indicated in Table I. These latter data were obtained with the use of an extract that had been dialyzed 4 hours against 0.001 M cysteine-0.001 M Tris buffer, pH 8.0, and the reaction was followed by colorimetric sulfhydryl estimation. Here, as in the case of pyruvate reduction, the requirement for pyridine nucleotide is not absolute, probably as a result of incomplete dialysis. The observed requirements, however, provide evidence for the conclusion that the over-all reaction

$$H_2 + GSSG \rightleftharpoons 2GSH \tag{4}$$

represents the sum of the following two events,

$$H_2 + TPN^+ \rightleftharpoons TPNH + H^+ \tag{5}$$

$$TPNH + H^+ + GSSG \rightleftharpoons 2GSH + TPN^+ \tag{6}$$

Determinations of K_m values and pH optima for coupled reactions of this type are valid only in so far as one of the pair can proceed at a rate which is not limiting the over-all process. For the system described above, with

[2] Prepared from bakers' yeast. The author is indebted to Dr. E. Racker for the unpublished procedure employed.

quantities of glutathione reductase 20-fold in excess of the requirement for the maximal rate of hydrogenase activity observed, the optimal pH was found to be 8.0. TPN saturates the system at 5×10^{-4} M; half saturation requires a concentration of 10^{-4} M. Higher concentrations (10^{-3} M) in-

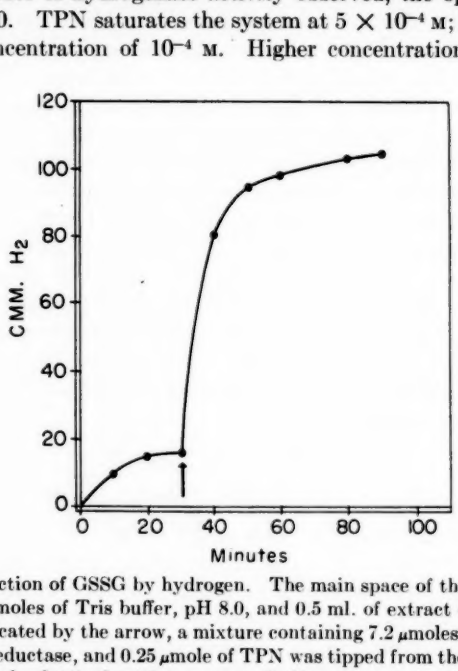


FIG. 3. Reduction of GSSG by hydrogen. The main space of the Warburg vessel contained 200 μmoles of Tris buffer, pH 8.0, and 0.5 ml. of extract dialyzed 3 hours. At the time indicated by the arrow, a mixture containing 7.2 μmoles of GSSG, 0.3 ml. of glutathione reductase, and 0.25 μmole of TPN was tipped from the side bulb. The center well contained 0.2 ml. of 20 per cent alkaline pyrogallol. Gas, H_2; temperature, 30°; final volume, 2.0 ml.

TABLE I

Requirements for GSSG Reduction by H_2

The complete system contained 100 μmoles of Tris buffer, pH 8.0, 0.25 μmole of TPN, 1.8 μmoles of GSSG, 0.2 ml. of glutathione reductase, and 0.2 ml. of extract dialyzed 4 hours. Incubation was carried out in stoppered test-tubes which had been flushed with hydrogen. Incubation time, 15 minutes at 30°. The values are in micromoles.

Components	GSH
Complete	1.32
GSSG omitted	0.03
Glutathione reductase omitted	0.03
C. kluyveri extract omitted	0.08
TPN omitted	0.30
H_2 omitted (He atmosphere)	0.06
Complete, 0 time	0.05

hibit the reaction. It was not established that this was attributable to TPN *per se* or to an impurity in the preparation employed. For the pyruvate system, saturation was achieved at 5×10^{-4} M DPN, and no inhibition was observed at higher concentrations.

Cofactor Requirement—The experiments described thus far were performed with extracts which were dialyzed for relatively short periods of time. More extensive dialysis (24 hours or longer) or ammonium sulfate precipitation resulted in a complete loss of activity. As much as 70 per cent of the activity was restored by the addition of small amounts of a boiled extract of whole cells or addition of reconcentrated dialysate, prepared either by vacuum distillation or by adsorption and elution from charcoal. In early attempts to investigate the nature of this additional requirement, an assay was employed based on the formation of reduced glutathione when extensively dialyzed enzyme was used. However, the impurity of the glutathione reductase employed rendered interpretations difficult, since it was noted that in an atmosphere of helium some reduction of GSSG occurred in the presence of boiled cell extract. Accordingly, an assay was designed to circumvent this difficulty, based on the reduction of pyruvate previously discussed, since lactic dehydrogenase of a reasonably high degree of purity can be prepared (5). The assay mixture contained 100 μmoles of Tris buffer, pH 8.0, 2.5 μmoles of potassium pyruvate, 0.5 μmols of DPN, 2000 units of lactic dehydrogenase, 2 μmoles of glutathione, dialyzed extract, and varying amounts of a source of "cofactor," in a final volume of 1 ml. Incubation was performed in test-tubes which were connected to a manifold and alternately evacuated to 0.05 atmosphere and refilled with hydrogen three times. After 15 minutes at 30°, the tubes were removed from the manifold, and the reaction was immediately stopped by the addition of 4 ml. of 6 per cent perchloric acid. The supernatant fluids were then analyzed for residual pyruvate. The effect of increasing amounts of boiled juice on pyruvate disappearance is demonstrated in Fig. 4. It will be seen that a saturation phenomenon is manifested; therefore a unit of cofactor activity may be defined as that amount which results in half maximal stimulation. These data were obtained with the use as cofactor of a boiled cell extract prepared as described below.

Cysteine and glutathione enhance the activity of hydrogenase, but only in the presence of boiled juice. However, cysteine was found to cause appreciable disappearance of pyruvate in an atmosphere of helium; glutathione alone may be satisfactorily used in the hydrogenase determination. It was found that ferrous ion activated this system; maximal activation requires 0.01 M Fe^{++}. This, however, appears to be as non-specific as the sulfhydryl activation, since Versene (0.02 M), dipyridyl (8×10^{-3} M), or *o*-phenanthroline (8×10^{-3} M) produces only 10 to 20 per cent inhibition

in the absence of added iron. As in the case of hydrogenase of *Proteus vulgaris* (9), storage under hydrogen was found not only to stabilize the enzyme, but to restore the activity to preparations which had declined in activity as a result of exposure to air. These observations suggest that some component of the enzyme must be in a reduced form to be catalytically active.

Properties of Factor—The chemical nature and mode of action of the factor or factors in the boiled cell extract are unknown. Of the biological

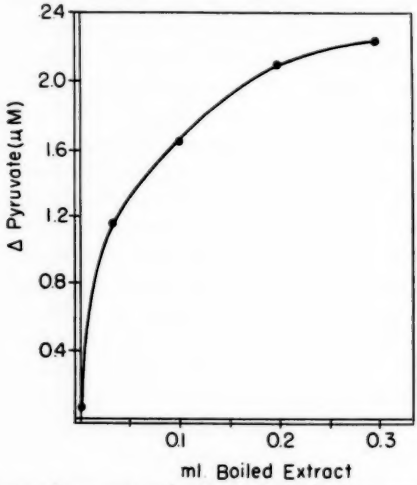


FIG. 4. Dependence of pyruvate reduction on boiled cell extract. 0.3 ml. of extract dialyzed 48 hours was employed as enzyme. Other conditions are described in the text.

materials thus far tested, *C. kluyveri* extract is by far the richest source. Wheat germ and Reticulogen are apparently devoid of activity.

With the assay described above, the following properties have been observed. (1) The factor is adsorbed by charcoal from aqueous solution at pH 2 to 3 and can be eluted with ammoniacal acetone. (2) It is similarly adsorbed on Florisil columns from acid solution and eluted with water or dilute neutral buffers. (3) In neutral solution, it is anionic, as evidenced by adsorption on Dowex 1 columns, but not by Dowex 50. (4) If acetone powders are prepared of cell-free extracts by conventional means (10 volumes of acetone), a considerable portion of the activity is not precipitated, but can be recovered from the supernatant solution after vacuum distillation. (5) It is difficultly extractable into ether from acid aqueous solution. (6) On paper chromatography, zones associated with activity are associated with ultraviolet absorption. No active fractions have been

obtained which are devoid of ultraviolet absorption in the region 250 to 260 mμ. The active zones in paper chromatograms contain no detectable phosphate when examined by the procedure of Bandurski and Axelrod (10).

A summary of the stability of the cofactor to acid, alkali, and peroxide appears in Table II.

That either two different factors or two interconvertible forms of the factor may exist is evidenced by the observations that two zones of activity separated by an inert zone are frequently encountered on paper chromatograms.

TABLE II

Stability of Hydrogenase Factor

To 0.5 ml. of boiled cell extract was added sufficient acid or alkali to give the final concentrations indicated in the table. After treatment, the acid samples were neutralized with KOH and the alkaline samples with HCl. Aliquots representing 0.05 ml. of boiled cell extract were assayed as described. To control samples equivalent amounts of KCl or NH_4Cl were added. The peroxide-treated samples were treated with catalase before assay.

Added agent	Final concentration	Temperature	Time	Per cent activity recovered
	N	°C.	*min.*	
HCl	0.1	25	30	95
"	0.1	100	5	65
"	1.0	25	30	60
"	1.0	100	5	10
NH_3	0.1	25	30	85
"	0.1	100	5	40
KOH	1.0	25	30	0
"	1.0	100	5	0
	per cent			
H_2O_2	3	25	15	80

The purine bases and their nucleotides cannot replace the factor requirement, nor can adenosine triphosphate, CoA, lipoic acid, or lipothiamide pyrophosphate. The last two compounds were tested after it was observed that the hydrogenase system is sensitive to arsenite. The arsenite inhibition is manifested when the acceptor for hydrogen is glutathione, pyruvate, or oxygen. A concentration of 10^{-3} M produces inhibition to 50 per cent of the control value.

Considerable variation is encountered in the activity of lots of *C. kluyveri* which have been grown separately. All lots tested, however, can be made essentially completely dependent on added cofactor. The major qualitative difference observed with different batches of enzyme is the nature of the response to enzyme concentration. While some enzyme preparations show essential linearity with enzyme concentration, others consistently

show a parabolic increase in activity with increasing enzyme concentration in the presence of saturating amounts of heat-stable factors (Fig. 5). This strongly suggests that more than one protein component is required for the reduction of pyridine nucleotides and that some preparations are sufficiently deficient in one enzyme component to make that component rate-limiting

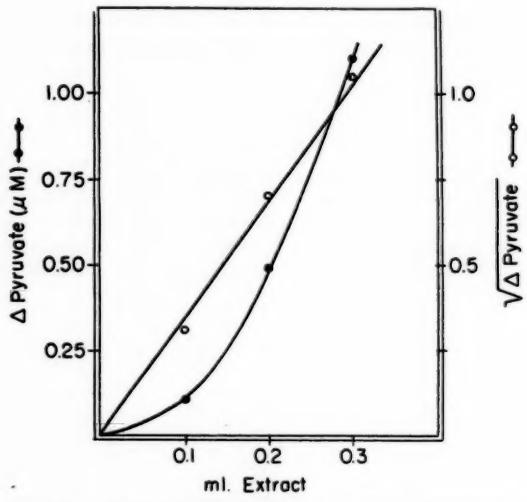


FIG. 5. Dependence of pyruvate reduction on enzyme concentration. The reaction mixture contained the following (in micromoles): Tris buffer, pH 8.0 (100), potassium pyruvate (2.5), DPN (0.5), and glutathione (2.0). Lactic dehydrogenase (2000 units) and boiled cell extract (0.3 ml.) were present in all tubes, in addition to the varying amounts of extract indicated in the graph. ●, observed pyruvate disappearance; ○, the square root of pyruvate disappearance. Final volume, 1.2 ml.; temperature, 30°.

for the over-all process, while in others two components are limiting and hence the rate of reaction is proportional to the square of extract concentration.

Attempts to demonstrate the reversibility of Reaction 2 have been unsuccessful. Under the conditions of growth on ethanol and acetate, the organism produces hydrogen as a fermentation product. This has been assumed to be coupled with the conversion of acetaldehyde to acetate, since this reaction is of sufficiently low oxidation-reduction potential to permit the concomitant evolution of hydrogen (11).

Since the potential of the glucose-gluconate half cell is slightly lower than that of the hydrogen electrode (12), it might be expected that with the proper catalysts the following over-all reaction should proceed from left to

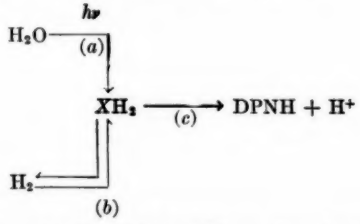
right,

$$\text{Glucose} \rightleftharpoons \text{gluconate} + H_2$$

Manometric experiments were performed with large amounts of glucose dehydrogenase and undialyzed *C. kluyveri* extract in the presence of glucose and DPN, in an atmosphere of helium. No gas evolution was observed during a 2 hour incubation period.

DISCUSSION

A relationship between photosynthesis and hydrogen utilization was first indicated by the observation of Gaffron (13) and Gaffron and Rubin (14) that algae could be adapted to utilize molecular hydrogen in place of light for reductive synthetic reactions. Production of hydrogen by exposure of adapted algae to light was also observed. Photoproduction of H_2 in photosynthetic bacteria has been observed by Gest and Kamen (15). These observations suggest that the processes of photosynthesis and hydrogen reduction may occur along common pathways after the initial step. Since photosynthetic activity has been observed to date only with particulate preparations, without apparent cofactor requirements, the present study was undertaken on the assumption that with the use of a soluble hydrogenase preparation required cofactors might be involved which could be common to both hydrogen utilization and photosynthesis. Vishniac and Ochoa (16) have succeeded in demonstrating photoreduction of pyridine nucleotides by isolated spinach grana. A sequence of events may be visualized linking these observations as follows:



Reaction *a* represents the chlorophyll-catalyzed transfer of hydrogen from water to a hydrogen acceptor, *X*, common to both pathways, Reaction *b* represents the action of hydrogenase, and Reaction *c* a common path operative in hydrogen bacteria and green plants for the reduction of pyridine nucleotides.

One possible conclusion that may be drawn from the failure to observe hydrogen evolution in the case of glucose and glucose dehydrogenase as a driving reaction is that *X*, which may represent the required factor for pyridine nucleotide reduction, is a substance of considerably lower oxidation-reduction potential than the DPN system.

Thus, if one assumes that the potential of XH_2 is reasonably close to the hydrogen electrode, no kinetic barrier would be encountered in the reduction of DPN by hydrogen. However, the DPN-mediated production of hydrogen from glucose would involve passage through an energy trough of considerable magnitude, approximately equivalent to the circumstances prevailing in a reduction of gluconate to glucose with reduced DPN. The over-all reaction, therefore, while thermodynamically feasible, would be expected to be extremely slow. Since the organism is capable of hydrogen liberation during growth, the possibility is suggested that some donor other than DPNH may transfer hydrogen directly to X in this process.

To date, no physiological reductant has been found for the conversion of crotonyl CoA to butyryl CoA. Extracts of *C. kluyveri*, however, can catalyze the formation of butyrate from acetyl phosphate and molecular hydrogen (2). The factor reported in this paper may represent a hydrogen carrier which is directly involved in the reduction of unsaturated to saturated fatty acids.

SUMMARY

Evidence is presented for the reduction of both DPN and TPN by molecular hydrogen, mediated by soluble extracts of *Clostridium kluyveri*. A heat-stable, alkali-labile factor is required for this process. Some of the properties of the system and of the cofactor are discussed.

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SULFHYDRYL AND PROSTHETIC GROUPS OF LACTIC OXIDATIVE DECARBOXYLASE FROM MYCOBACTERIUM PHLEI

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In a previous paper (1), conditions were described for the isolation and partial purification of a lactic oxidative decarboxylase from *Mycobacterium phlei*. For each mole of lactate metabolized by this enzyme, 1 mole of oxygen is utilized and 1 mole each of acetate and CO_2 is formed. The present paper deals with additional properties of the enzyme, the possible nature of the prosthetic group, and the evidence for the presence of one or more sulfhydryl groups.

Materials and Methods

The method for the purification of the crude enzyme extract has been described previously (1). Special reagents were obtained as follows: FAD[1] (30 per cent) and PCMPS, Sigma; FMN, California Foundation for Biochemical Research. Spectrographic analyses were made in a Bausch and Lomb 1.5 meter stigmatic grating spectrograph. Electrophoretic data were obtained in a Tiselius apparatus equipped with a cylindrical lens optical system.

Results

Further Purification of Enzyme

Electrophoresis—Schlieren patterns of the lactic oxidative decarboxylase, fractionated as described previously (1), demonstrated that the enzyme preparation contained one major and several minor components (Table I). Samples withdrawn from the electrophoresis cell and tested for enzymatic activity confirmed the conclusion that the major ascending component contained the specific activity. The minor ascending components were enzymatically inactive. These observations suggested that removal of the minor components was desirable before further investigation of the enzyme should be undertaken.

Fractionation—The enzyme preparations previously described were essentially colorless after the final dialysis against distilled water (1). A

[1] The following abbreviations are used: FAD, flavin adenine dinucleotide; FMN, riboflavin-5′-phosphate; PCMPS, *p*-chloromercuriphenylsulfonate; PCMB, *p*-chloromercuribenzoate; Tris, tris(hydroxymethyl)aminomethane.

preparation resulting from the extraction of 5 times the initial amount of cell material previously used had a distinct brown color. The absorption spectrum was determined in the Cary recording spectrophotometer. In place of a low order of general absorption in the range 330 to 440 mμ,[2] a distinct peak at 410 mμ was noted. This result suggested a possible relationship between the minor components observed electrophoretically in Preparations 1 and 2 (Table I) and the material absorbing at 410 mμ.

Attempts to remove these minor protein components by adsorption or by ammonium sulfate fractionation failed. The separation of the material

TABLE I

Electrophoretic Mobility Data of Lactic Oxidative Decarboxylase from M. phlei

Preparation No.	Buffer system	Ionic strength	Time	Mobility × 10^{-5}*
			min.	*cm.² per volt per sec.*
1	Phosphate, pH 6.9	0.060	195	
2	" " 6.0	0.038	115	
3	" " 5.9	0.100	360	+8.50
4a†	" " 5.9	0.100	330	+8.25
4b†	" " 5.9	0.100	300	+8.34
5‡	" " 6.9	0.100	360	+10.0
6‡	" " 7.7	0.100	240	+10.6
7‡	Acetate, pH 5.4	0.100	140	+5.80

All enzyme preparations were brought to equilibrium with buffer solution by dialysis at 4° for 24 hours before the determinations were made.

* Mobility of major ascending component.

† Combined major ascending components withdrawn from cell following electrophoresis of partly purified enzyme.

‡ Acetate-fractionated enzyme material. For details see the text.

absorbing at 410 mμ from the active enzyme was accomplished by the use of solubility differences at various acidic pH levels. The most efficient separation occurred when the enzyme preparation was dialyzed against 0.05 M acetate buffer, pH 5.1, for 18 hours at 4°. The precipitated protein was removed by centrifugation and then suspended in 10 ml. of fresh acetate buffer. After centrifugation, the packed protein was suspended in 0.1 M phosphate buffer, pH 6.0. The resulting supernatant solution, after centrifugation, contained the enzyme. The recovered activity was 40 per cent of the original starting material and the specific activity, expressed as units per mg. of protein, was increased 1.5-fold.

The purified enzyme was examined electrophoretically and found to con-

[2] Fig. 4 of Sutton (1).

sist of a single major component accompanied by a small minor component (Preparation 5, Table I). Most of this minor component, as well as of the other minor components described above, was removed by the purification procedure. Samples of the purified enzyme were examined at various pH levels in the Tiselius apparatus. At pH levels between 4.5 and 5.0, the enzyme was not sufficiently soluble to permit determinations to be made. The enzyme was irreversibly denatured at more acidic pH levels.

Properties of Purified Enzyme

Sulfhydryl Group—Neilands (2) has reported the inhibition of lactic dehydrogenase from heart muscle by PCMB but not with iodoacetate (3). The lactic oxidative decarboxylase is not inhibited by iodoacetate (1). These facts suggested that the presence of possible sulfhydryl groups in the purified enzyme should be investigated.

Inhibition experiments with various sulfhydryl-binding reagents were performed in the Warburg apparatus. The mercaptide-forming agents PCMB and PCMPS at a final concentration of 6×10^{-4} M decrease the enzyme activity by 93 and 97 per cent, respectively. No significant inhibition was observed with either *o*-iodosobenzoate or iodoacetate. The extent of CO_2 production in these experiments was of the same order as that for the oxygen utilization.

Barron (4) points out that the protection of enzyme sulfhydryl groups from inhibition by substrates or coenzymes is characteristic of certain enzymes. Substrate protection was not observed with the lactic oxidative decarboxylase. A gradually increasing inhibition over a 15 minute period when PCMPS was added after the reaction was in progress suggests that the substrate possibly may be attached to the enzyme through a sulfhydryl group.

No reversal of the PCMPS inhibition was observed when L-cysteine, glutathione, or ethylenediaminetetraacetic acid was added to the reaction mixture 15 minutes after substrate addition. The degree of reversal of the inhibition of sulfhydryl-binding reagents is determined by the affinity of the enzyme and the reversing agent for the mercaptide-forming compounds (4).

It will be seen that preincubation of an excess of cysteine with PCMB before addition of the enzyme failed to prevent inhibition (Table II). The extent of the inhibition was not as great as that produced by the same concentration of PCMB (Experiments 1 and 2, Table II). The spectrophotometric technique described by Boyer (6) showed that mercaptide formation, i.e., cysteine plus PCMB, is complete within the 15 minute preincubation period used in the above experiments. This fact demonstrates that PCMB would not be available for enzyme inhibition unless the

affinity of the enzyme is greater than that of cysteine. Similar experimental results were obtained with PCMPS and cysteine or glutathione (Experiment 3, Table II).

TABLE II

Relative Affinity of Enzyme Sulfhydryl Groups Versus Cysteine (or Glutathione) for p-Chloromercuriphenylsulfonate (or p-Chloromercuribenzoate)

Experiment No.	Additions	Oxygen utilization*	Per cent inhibition
		μl.	
1	None	41	
	PCMB (2.6×10^{-4} M)	16	61
	" (2.6×10^{-4} ") + L-cysteine (1.85×10^{-3} M)†	23	44
	L-Cysteine (1.85×10^{-3} M)	38	
2	None	45	
	PCMB (6×10^{-4} M)	3	93
	" (6×10^{-4} ") + L-cysteine (1.85×10^{-3} M)†	13	72
3	None	52	
	PCMPS (6×10^{-4} M)‡	7	87
	" (6×10^{-5} ")	39	25
	" (6×10^{-4} ") + L-cysteine (6×10^{-4} M)†	30	42
	" (6×10^{-5} ") + " (6×10^{-4} ")†	50	4
	" (6×10^{-4} ") + glutathione (6×10^{-4} M)†	42	19
	" (6×10^{-5} ") + " (6×10^{-4} ")†	51	2
	Glutathione (6×10^{-4} M)	51	2
	L-Cysteine (6×10^{-4} ")	51	2

Purified enzyme, Experiments 1 and 2 (specific activity 50), 62 γ of protein per Warburg vessel; Experiment 3 (specific activity 197), 81 γ of protein per Warburg vessel; 0.5 ml. of 0.4 M lithium lactate solution; 0.5 ml. of 0.1 M phosphate buffer, pH 5.9; gas oxygen; temperature 37°; final volume 2.0 ml. All concentrations given as final molarity.

* Oxygen utilization for the second 10 minute period of the reaction.

† PCMB (or PCMPS) preincubated with L-cysteine (or glutathione) for 15 minutes before addition of enzyme.

‡ PCMPS was used in place of PCMB because of its better solubility properties (5). PCMPS was dissolved in 1 M phosphate buffer, pH 5.9.

Hellerman and Frisell (7) have reported that PCMB is a competitive inhibitor for D-amino acid oxidase. The benzoate portion of the PCMB molecule functions as an inhibitor independently of the mercury group. The effect of benzoate, *m*-toluate, and $HgCl_2$ on the lactic oxidative decarboxylase was studied. No inhibition was observed except with $HgCl_2$. This effect is very likely due to either the combination of the $HgCl_2$ with the amino and carboxyl groups of the enzyme protein (8) or the reaction of the

compound with dithiol groups (9). This latter possibility seems less likely in light of the failure of *o*-iodosobenzoate to inhibit the enzyme.

Prosthetic Group—The yellow color of the purified enzyme, which was apparent after the removal of the brown protein component, suggested the presence of a functional chromophore group. The absorption spectrum of the enzyme indicated that a riboflavin-containing compound might account for the color. The presence of FAD in the lactic oxidative decarboxylase had not been detected (1). The evidence consisted of chromatographic analysis for adenine after perchloric acid hydrolysis and the absorption spectrum of the partly purified enzyme. In the absence of the brown, quenching component, absorbing at 410 mμ, the purified enzyme gave peaks at 275, 375, and 450 mμ. This result suggested a need for investigating the relationship between the yellow color and the activity of the enzyme, particularly in light of the level of specific activity of the colorless, electrophoretically fractionated material. The specific activities of the electrophoretic samples were higher than those of the yellow enzyme preparation.

The purified enzyme was treated with an acetone-hydrochloric acid solution in a manner similar to that described by Theorell (10). The acidified acetone was removed under vacuum, and the remaining residue was dissolved in a minimal quantity of distilled water. A second aliquot of the enzyme preparation was treated with $(NH_4)_2SO_4$-H_2SO_4 according to the method described by Warburg and Christian (11). The resulting protein-free solution was concentrated under vacuum and then neutralized by the addition of 0.5 M dibasic phosphate solution.

These extracts were examined by descending paper strip chromatography. Typical results are recorded in Table III. A single fluorescent spot was found in each of the three solvent systems used. These results are strong evidence against degradation of the yellow component during removal from the enzyme protein. The mobility of the unknown material in the various solvent systems nearly corresponds to FMN, although slightly different R_F values were obtained (Table III).

The acetone-HCl extract gave a typical flavin absorption spectrum with peaks at 265, 375, and 450 mμ. The peak ratios of the $(NH_4)_2SO_4$-H_2SO_4 extract suggest that the compound in question is closely related, if not identical, to FMN (Table IV). Both extracts gave the same R_F values on chromatograms. The reasons for the higher ratios in the case of the acetone-HCl extract are not apparent.

The $(NH_4)_2SO_4$-H_2SO_4 extract was analyzed fluorometrically (16). The nucleotide pyrophosphatase of Kornberg and Pricer (17) was employed in an attempt to increase the fluorescence of the unknown compound. No increase in fluorescence was observed. In addition, it was noted that the

TABLE III

Paper Chromatography Data of Flavin Extract from Purified Lactic Oxidative Decarboxylase

Flavin	Solvent System 1*	Solvent System 2*	Solvent System 3*
	R_F	R_F	R_F
Riboflavin	0.539	0.594	0.325
FAD	0.103	0.010	0.410
FMN	0.355	0.008	0.552
Acetone-HCl extract†	0.380	0.012	0.561

All flavins applied to paper strips in approximately the same concentration. The ascending flavin spots were observed for fluorescence with a Mineralight No. SL2537 operating at 2537 A. All chromatography experiments were run with light excluded.

* Solvent System 1, *tert*-amyl alcohol (3 parts), formic acid (1 part), and water (1 part) according to Hanes and Isherwood (12). Time 7 hours; temperature 16°. Solvent System 2, *tert*-butyl alcohol (3 parts), pyridine (3 parts), and water (1 part) according to Crammer (13). Time 20 hours; temperature 16°. Solvent System 3, 5 per cent Na_2HPO_4 in water saturated with isoamyl alcohol according to Carter (14). Time 6 hours; temperature 16°.

† For preparation of acetone-HCl extract see the text. $(NH_4)_2SO_4$-H_2SO_4 extract gave similar results.

TABLE IV

Ratio of Light Absorption of Enzyme Flavin Extract, FAD, FMN, and Riboflavin at Different Wave-Lengths

Flavin	$\frac{260\ m\mu}{450\ m\mu}$	$\frac{275\ m\mu}{450\ m\mu}$	$\frac{375\ m\mu}{450\ m\mu}$
Acetone-HCl extract A*	5.0	3.0	0.9
" " B*	4.5	3.8	0.9
" " C†	3.4	2.6	1.2
$(NH_4)_2SO_4$-H_2SO_4 extract‡	2.6	1.9	0.9
FAD (90% pure)§	3.3	2.5	0.8
" (30% ")‖	3.5	2.7	0.8
FMN‖	2.1	2.0	0.9
Riboflavin‖	2.2	2.1	0.9

* Extracts from different oxidative decarboxylase preparations. See the text for the method of preparation.

† Inorganic phosphate-free acetone-HCl extract. See the text for the method of preparation.

‡ See the text for preparation of the extract.

§ According to Whitby (15).

‖ All values calculated from readings in the Beckman model DU spectrophotometer.

yellow color of both extracts was removed on treatment with either sodium borohydride or dithionite.

Reversible Splitting—The enzyme was split according to the method described by Horecker (18). The residual activity of the enzyme protein was increased by the addition of FAD, FMN, and riboflavin (Experiment 1, Table V). The acetone-HCl extract described above failed to increase the residual activity of the enzyme protein under the conditions of the experiment. It is apparent from the experimental results that a lack of specificity exists between the enzyme protein and the added flavins. A similar lack of specificity of enzyme protein for its natural flavin prosthetic group has been reported by Horecker (18) and others (19).

The largest reactivation per mole of added flavin was obtained with FMN (Table V). The failure of the acetone-HCl extract to reactivate the enzyme protein at a level of 0.012 μmole suggested the presence of

TABLE V

Reactivation of Lactic Oxidative Decarboxylase Protein by Flavins in Presence of Phosphate Buffer

Additions		Oxygen utilized*	
		Experiment 1	Experiment 2
	μmole†	μl.	μl.
None		194‡	159‡
FAD	0.025	232	
"	0.039	238	
FMN	0.007		122
"	0.009	247	
"	0.014	257	140
Riboflavin	0.020	215	
Acetone-HCl extract§	0.006	187	
" "	0.012	103	
$(NH_4)_2SO_4$-H_2SO_4 extract§	0.002		75
" "	0.004		64
" "	0.009		45

Each Warburg vessel contained 66 γ of lactic oxidative decarboxylase protein in addition to 0.5 ml. of 0.4 M lithium lactate solution, 0.4 ml. of 0.1 M phosphate buffer, pH 6.0 (Experiment 1), 0.4 ml. of 1.0 M phosphate buffer, pH 6.0 (Experiment 2); gas oxygen; final volume 2.0 ml.; temperature 37°; time 30 minutes.

* Enzyme protein preincubated with flavin for 30 minutes before substrate addition.

† Calculated from Beckman spectrophotometer data at 450 mμ.

‡ The enzyme protein preparation lost some residual activity while held in a frozen state between Experiments 1 and 2.

§ Calculated as FMN.

an inhibitory substance. This extract had been previously demonstrated to have different absorption peak ratios from those obtained with the $(NH_4)_2SO_4$-H_2SO_4 extract (Table IV). A similar experiment was performed with this latter extract (Experiment 2, Table V). The data indicate that the $(NH_4)_2SO_4$-H_2SO_4 extract was more inhibitory (Experiment 1, Table V). In addition, added FMN failed to reactivate the same enzyme protein used in Experiment 1 (Table V). The only change in the experimental procedure was the use of 1.0 M in place of 0.1 M phosphate buffer. These results suggest that an increased buffer concentration in some manner prevented the reactivation of the enzyme protein.

The reactivation experiment was performed with the same sample of enzyme protein with FMN (0.014 μmole) and 0.05 M Tris buffer, pH 6.9, as well as 1.0 M phosphate buffer, pH 6.8, and 0.1 M phosphate buffer, pH 6.8. The results indicate that in the absence of inorganic phosphate the reactivation of the enzyme protein is more extensive. The molar concentration of the inorganic phosphate present also influences the degree of reactivation.

A sample of the purified enzyme in 0.1 M phosphate buffer was dialyzed against distilled water to remove inorganic phosphate. The dialyzed material was treated as previously indicated, except that the enzyme protein was dissolved in 0.05 M Tris buffer, pH 6.9. An acetone-HCl extract was prepared from the dialyzed enzyme material.

Experiments designed to demonstrate an increase in residual activity of the enzyme protein were performed (Experiment 1, Table VI). FAD failed to increase the residual activity at concentrations which previously had produced an increase in the system containing inorganic phosphate (Experiment 1, Table V). Riboflavin was apparently slightly inhibitory. FMN increased the residual activity of the enzyme protein (Experiments 1 and 2, Table VI). The inorganic phosphate-free acetone-HCl extract did not increase the residual activity of the enzyme protein.

Absorption Spectrum of Enzyme—The nature of the flavin group in the enzyme suggested that spectral changes might be demonstrated in the presence of the specific substrate. The absorption spectrum in the presence and absence of substrate was determined in the Cary recording spectrophotometer. The difference spectrum, *i.e.*, oxidized minus reduced, shows that the peaks at 375 and 450 mμ are associated with riboflavin-containing compounds.

Oxidation-Reduction Potential—The lactic oxidative decarboxylase has not been previously shown to reduce either neotetrazolium[3] or methylene blue (1). This fact indicates that the potential of the enzymatic system is relatively positive. In the absence of associated metal ions, demon-

[3] 2,2-(*p*-Diphenylene)bis(3,5-diphenyl)tetrazolium chloride.

strated by spectrographic analysis and the failure of chelating agents to influence the enzymatic reaction, a single electron transport system appears unlikely.

The enzyme-substrate reaction of the purified lactic oxidative decarboxylase was studied in the presence of the following oxidation-reduction indicators: 2,6-dichlorophenolindophenol (E'_0 at pH 7.0, +0.217), *o*-cresolindophenol (E'_0 at pH 7.0, +191), and toluylene blue (E'_0 at pH 7.0, +0.115) (20). 2,6-Dichlorophenolindophenol served as an electron acceptor. The potential of the enzyme-substrate system is estimated to be more positive than +0.191 volt. It should be noted that the potential of the "old" yellow enzyme of Warburg has been reported to be −0.06 volt (21).

Oxygen Requirement—As previously reported (1), the rate of oxygen utilization and CO_2 production for the enzyme reaction is not directly proportional to the enzyme concentration. Preparations with high levels of specific activity exhibited this under a variety of conditions and enzyme concentrations. These facts suggested that a rate-limiting factor was responsible. Experiments were conducted in which the concentration of oxygen in the reaction mixture was varied by increasing the shaking rate of the Warburg vessel or by gassing the vessel with pure oxygen. The data obtained demonstrate that a direct proportionality does exist between the enzyme concentration and the rate of oxygen utilization.[4] The same relationship holds for the CO_2 production. A proportional relationship between oxygen utilization and enzyme concentration was only apparent at low enzyme concentrations and in the presence of pure oxygen.

The average turnover number calculated for several electrophoretically fractionated enzyme preparations is 470 moles of lactate per mole of enzyme per minute in the presence of oxygen, with an assumed enzyme molecular weight of 75,000. This latter value is based on the figures determined for other flavoproteins (18). It is of interest to note that the turnover number of Warburg's "old" yellow enzyme is reported to be 50 (22).

DISCUSSION

In contrast to the colorless enzyme preparations previously obtained (1), a preparation resulting from the extraction of 5 times the initial amount of cell material had a distinct brown color and gave an absorption maxi-

[4] The unit of enzyme activity has been redefined (1) in light of the experimental data presented in this paper. In the presence of pure oxygen a proportionality between the concentration of the enzyme and the oxygen utilization has been established. The unit of enzyme activity has therefore been defined as that amount of enzyme that is sufficient to produce 10 μl. of gas exchange in 10 minutes, calculated from the second 10 minutes of the reaction.

mum at 410 mμ. A search of the literature revealed that troublesome brown hemin pigments, absorbing at 405 to 415 mμ, are frequently encountered during enzyme purification (18, 23). In addition, these pigments obscure the flavin spectrum of the enzyme with which they are associated (18, 23, 24). In the absence of the brown pigment the lactic oxidative decarboxylase preparation was bright yellow and gave a typical flavoprotein absorption spectrum.

It was evident at this point in the investigation that the possible means of electron transport from the substrate to oxygen was at hand. All previous attempts to determine the nature of the prosthetic group associated with the lactic oxidative decarboxylase had failed (1). The spectral changes occurring in the presence of substrate established the relationship of the flavin to the enzyme activity. The failure to demonstrate a typical flavoprotein absorption spectrum despite high levels of specific activity (1), as well as the absence of color in the electrophoretically purified preparations (Preparations 4a and 4b, Table I), requires explanation before the above relationship can be acceptable.

The quenching of the spectrum by the component at 410 mμ need not be considered, since this protein had been removed from the electrophoretically fractionated preparations. The high level of specific activity of these preparations in the absence of sufficient flavin to produce an absorption spectrum can only be explained on the basis of concentration, *i.e.*, units of enzyme activity. Haas *et al.* (24, 25) have reported a similar experience during the isolation and purification of cytochrome *c* reductase from yeast. It has been determined that an excess of 300 units[4] per ml. of the lactic oxidative decarboxylase must be present for the yellow color, as well as the absorption spectrum, to be detected. In light of this fact the relationship between the spectral changes and the enzyme activity becomes acceptable.

The lack of specificity between a natural flavin prosthetic group and its enzyme protein has been reported (18, 19). The experimental results presented in Table VI suggest that the presence of inorganic phosphate in the reaction mixture may influence this specificity.[5]

The fact that 2,6-dichlorophenolindophenol acts as an electron acceptor,

[5] After submission of this paper for publication, similar observations on the effect of phosphate on the reactivation of the "old" yellow enzyme protein by FMN were reported by Theorell and Nygaard (26). These authors have studied the effect of monovalent and polyvalent anions on the association and dissociation of the FMN-enzyme protein complex. Phosphate was found to decrease the association velocity and chloride to increase the dissociation. The presence of chloride ions in the acetone-hydrochloric acid extract (Table VI) may account for the failure to observe reactivation of the protein of the lactic oxidative decarboxylase with this preparation.

but that *o*-cresolindophenol does not, indicates that the potential of the system is relatively positive. The absence of demonstrable metal ions and the failure of cyanide to inhibit the enzyme reaction (1) suggest the presence of a 2 electron transport system. The presence of FMN, or an FMN-like substance, in the enzyme strengthens this view, with the possible exception that the formation of H_2O_2 as a reaction product has not been

TABLE VI

Reactivation of Lactic Oxidative Decarboxylase Protein by Flavins in Inorganic Phosphate-Free System

Additions		Oxygen utilized*	
		Experiment 1	Experiment 2
	μmole†	μl.	μl.
None		182‡	175‡
FAD	0.020	182	
"	0.039		147
FMN	0.007	196	
"	0.008		189
"	0.014	211	
"	0.017		205
Riboflavin	0.020	160	145
Acetone-HCl extract§	0.006		174
" "	0.012		173

Each Warburg vessel contained 56 γ of lactic oxidative decarboxylase protein (inorganic phosphate-free) in addition to 0.5 ml. of 0.4 M lithium lactate solution, 0.4 ml. of 0.05 M Tris buffer, pH 6.9; gas oxygen; temperature 37°; final volume 2.0 ml.; time 30 minutes.

* Enzyme protein preincubated with flavin for 30 minutes before substrate addition.

† Calculated from Beckman spectrophotometer data at 450 mμ.

‡ The enzyme protein preparation lost some residual activity while held in a frozen state between Experiments 1 and 2.

§ Calculated as FMN. Extract inorganic phosphate-free.

demonstrated (1). The high level of activity in the presence of oxygen suggests a rapid turnover rate and excludes the necessity of postulating a second catalyst in addition to the autoxidizable flavin.

The degree of reversal of the inhibition of essential sulfhydryl groups by sulfhydryl-binding reagents is determined by the affinity of the enzyme sulfhydryl groups and the thiol-reversing agent for the inhibitor. Reactivation should be complete, if sufficient concentration of thiol is added, unless irreversible denaturation has occurred (4). In certain cases the failure to obtain reversal is attributed to other reactions. Mahler and Elowe (27) were not able to demonstrate reactivation of DPN-cytochrome

c reductase inhibited with PCMB. These workers have indicated that the inhibitor exerts its effect at a site other than that of a sensitive sulfhydryl group.

Experiments were designed to test the affinity of the lactic oxidative decarboxylase sulfhydryl groups for PCMPS and PCMB (Table II). The manner in which these experiments were performed should result in complete removal of all available inhibitor. The results clearly indicate that the affinity of the enzyme sulfhydryl groups for the inhibitor is greater than that of the thiol-reversing agents, except when a 10-fold concentration of glutathione was used (Table II). In this case the lack of affinity of the enzyme for PCMPS indicates that complete reversal of the inhibition of the enzyme should be possible. The fact that no reversal was obtained when thiols were added after the inhibitor, and yet affinity experiments show that reversal is possible (Table II), indicates that essential sulfhydryl groups may be present, but their demonstration by the usual reversing experiments is not possible because of the nature of the reaction between the inhibitor and the enzyme protein.

SUMMARY

The lactic oxidative decarboxylase from *Mycobacterium phlei* has been isolated in a highly purified form after removal of minor protein components. The purity of the enzyme has been established by electrophoretic studies.

The yellow enzyme preparations show a typical flavoprotein spectrum, but electrophoretically fractionated preparations with higher levels of specific activity are colorless. Evidence obtained from paper chromatography and reactivation experiments indicates that the prosthetic group is a flavin similar to, if not identical with, riboflavin-5′-phosphate. The effect of inorganic phosphate on the reactivation of the enzyme protein with flavins has been studied.[5]

Although experiments designed to demonstrate the presence of sulfhydryl groups in the enzyme were not conclusive, the existence of these groups has not been completely excluded.

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THE AMINO ACID REQUIREMENTS OF MAN

XIII. THE SPARING EFFECT OF CYSTINE ON THE METHIONINE REQUIREMENT*

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In Paper XI of this series (1), consideration was given to the problem of the methionine requirement of man as measured by the maintenance of nitrogen equilibrium. Quantitative data obtained in six male subjects demonstrated that the minimal needs varied from 0.8 to 1.1 gm. daily. D-Methionine, in contrast to the behavior in man of the D isomers of all other indispensable amino acids, was found to be just as effective as DL-methionine for the purpose in question. From this observation, it follows that L-methionine does not possess a measurably greater metabolic activity than does the racemic compound, and that the methionine requirement of an individual is the same whether expressed in terms of the L- or the DL-amino acid.

In accordance with the practice followed in most of our human investigations, the diets employed in the above experiments contained the eight essential amino acids (*cf.* (2)), with added glycine and urea as sources of nitrogen for the synthesis of the non-essentials. Consequently, the subjects were deprived of cystine. In view of the metabolic relationship of methionine and cystine (3–6), one would anticipate that the inclusion of the latter in the food would affect the needs of the organism for the former. Indeed, the sparing action of cystine upon the methionine requirement of animals can be demonstrated readily. Several years ago, Womack and Rose (7) concluded that weanling rats require 0.6 per cent of DL-methionine for maximal gains when the diets are devoid of cystine, and that the addition to the food of moderate quantities of L-cystine reduces the methionine requirement to 0.5 per cent. These findings appeared to indicate that, in the rat, one-sixth of the minimal methionine needs for growth could be satisfied by cystine.

The experiments of Womack and Rose antedated the commercial pro-

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duction of most of the vitamins of the B complex; consequently, at that time, the best available sources of these dietary factors were concentrates prepared from rice polishings, milk, or yeast. With the advent of abundant supplies of crystalline vitamins, the earlier tests were repeated under more ideal circumstances. Wretlind and Rose (8) showed that, with diets containing 0.2 per cent of L-cystine, maximal growth could be obtained with 0.4 per cent of L-methionine. Furthermore, in the growing rat as in the adult man, no significant difference could be detected in the effectiveness of the optical isomers of methionine. Unpublished experiments involving the use of rations which were devoid of cystine have served to verify the original finding of Womack and Rose; namely, that 0.6 per cent of methionine is the minimal amount which is capable of inducing maximal gains. In the light of the newer data, it is evident that approximately one-third of the methionine requirement of the growing rat can be satisfied by cystine.

The investigation herein described had as its objective a determination of the sparing effect of cystine upon the methionine requirement of adult man. This is a problem of more than passing interest since, in certain areas of the world, methionine is more likely than any other amino acid to be a limiting component of the foods commonly consumed (*cf.* Malhus *et al.* (9)). Therefore, to the extent that cystine is present in such dietaries, it may play an amazingly significant rôle, as will be revealed by the experiments outlined in the following pages.

EXPERIMENTAL

As in all of the investigations of this series, normal young men served as the subjects. The diets were analogous to those previously described and were composed of mixtures of highly purified amino acids, wafers, extra sucrose and butter fat, and Cellu flour to provide a residue in the alimentary tract. The amino acids were taken in aqueous solution flavored with filtered lemon juice and sucrose. The wafers furnished most of the energy and all of the inorganic salts. Vitamins were administered once or twice daily in the form of a concentrate of fish liver oil and supplements carrying the other accessory factors.[1] One-third of the daily allotment of amino

[1] A gradual change has occurred in the procedure followed in making the vitamin pills (10). In certain experiments, without modifying the kind and quantities of the active components, approximately 5 gm. of dextrin have been used each day as a diluent and binder. In later experiments, to disguise more completely the flavor of the liver concentrate, larger quantities of dextrin or sucrose, or both, have been used. Under these circumstances, the products have ceased to be pills and must be eaten rather than swallowed whole. The calories contributed by the carbohydrates have been included, of course, in calculating the energy intakes of the subjects. Obviously, this variation in the method of administering the vitamins has not affected the outcome of the experiments. The details are furnished merely as an aid to those who may conduct similar investigations.

acids, wafers, and extra sucrose and butter fat was consumed at each meal. The reader is referred to Paper I of this series (10) for the composition of the salt mixture and baking powder used in making the wafers, the daily intakes of the vitamins, and other pertinent information concerning the conduct of the experiments.

In order to determine the effect of cystine, it was necessary, first of all, to establish the minimal methionine requirement of each subject when cystine was absent from the food. Once this had been accomplished, a uniform daily dose of L-cystine was administered and the methionine requirement was again measured. Throughout, the total nitrogen intake of each subject was maintained at a constant level by suitable adjustments in the amount of glycine present in the amino acid mixture. DL-Methionine was used in all experiments since its effectiveness is not measurably different from that of the L isomer (1).

Three experiments were carried out, and the results obtained were in close agreement. Subject R. L. W. received initially a diet consisting of amino acid Mixture 205 (the composition of which was described in the preceding paper (11) of this series), Wafers IV (*cf.* Rose *et al.* (12)), 301.3 gm. of extra sucrose, and 42.4 gm. of extra butter fat. The daily intake of amino acid Mixture 205 furnished 9.70 gm. of nitrogen, of which 0.44 gm. was derived from the D isomers of valine, isoleucine, and threonine. The nitrogen intake from all sources was 10.10 gm. per day, of which 0.40 gm. was of an unknown nature. The latter originated largely in the starch of the wafers. Much smaller amounts were contributed by the butter fat, lemon juice, and liver concentrate present in the vitamin pills. The daily energy intake amounted to 55 calories per kilo of body weight. In this experiment, as well as in the other two, the ratio of calories derived from carbohydrates to those furnished by fats was maintained at 2.6.

Mention should be made of the fact that the amount of extra sucrose consumed by Subject R. L. W. was larger than has been used in other experiments. This subject weighed in excess of 80 kilos. In order to provide the desired energy intake, without modifying the customary ratio of calories originating in carbohydrates and fats, an increase was necessary either in the starch of the wafers or in the extra sucrose. The latter alternative was chosen by the subject in order thereby to avoid an increase in the *bulk* of wafers to be ingested. That the sucrose was completely utilized was demonstrated by frequent tests of the urines for reducing materials, before and after boiling the samples with acid. Invariably the tests were negative. Indeed, tests for sugar and protein were made routinely throughout all of our human experiments.

The findings in the experiment upon Subject R. L. W. are summarized in Table I. Starting with a daily dosage of 2.0 gm. of DL-methionine, a strongly positive nitrogen balance was established promptly and was main-

tained throughout the period. Distinctly positive balances also occurred during the two suceeding periods, when the daily methionine intakes were 1.0 and 0.9 gm., respectively. On the other hand, further decreases resulted in negative balances. Even during the fourth period, when the

TABLE I

Sparing Effect of Cystine on Methionine Requirement of Man

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
			Subject R. L. W.			
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
6	81.9	10.10	8.82	0.92	+0.36	2.0 gm. DL-methionine
7	82.6	10.10	8.89	0.89	+0.32	1.0 " "
6	82.7	10.10	8.92	0.88	+0.30	0.9 " "
6	83.1	10.10	9.19	0.92	−0.01	0.8 " "
4	84.1	10.10	9.39	0.84	−0.13	0.7 " "
6	83.6	10.10	8.54	0.77	+0.79	0.8 " " 0.81 gm. L-cystine†
6	84.7	10.10	8.40	0.71	+0.99	0.4 gm. DL-methionine; 0.81 gm. L-cystine
7	85.1	10.10	8.97	0.75	+0.38	0.1 gm. DL-methionine; 0.81 gm. L-cystine
6	85.2	10.10	9.05	0.80	+0.25	0.1 gm. DL-methionine; 0.81 gm. L-cystine
7	85.3	10.10	9.38	0.77	−0.05	No DL-methionine; 0.81 gm. L-cystine
4	85.2	10.10	9.46	0.79	−0.15	No DL-methionine; 0.81 gm. L-cystine
6	85.3	10.08‡	8.73	0.74	+0.61	0.1 gm. DL-methionine; 0.81 gm. L-cystine

* The initial diet contained amino acid Mixture 205 (11).

† Equivalent in sulfur content to 1.0 gm. of methionine.

‡ The slight decrease in nitrogen intake during this period was due to the use in the wafers of a new shipment of starch which had a slightly lower nitrogen content.

average state of the balance indicated almost exact equilibrium, moderate losses of body nitrogen occurred on 3 of the 6 days. Clearly, the minimal requirement of this subject, in the absence of dietary L-cystine, was 0.9 gm. daily.

With the information thus acquired, it was now possible to determine the influence of cystine. For this purpose, the methionine dosage was raised to 0.8 gm., and the resulting ration was supplemented by the daily addition

of 0.81 gm. of L-cystine. The latter quantity contains the same amount of sulfur as that present in 1.0 gm. of methionine. Thus, the cystine intake was more than equivalent to the methionine actually needed by the subject on a cystine-free diet. As the data in Table I indicate, the effect of this diet was extraordinary. Immediately, the nitrogen balance became strongly positive, with an average daily retention of 0.79 gm. This situation persisted during the next period, when the methionine content of the food was reduced to 0.4 gm. daily. But even more astounding is the fact that a daily methionine intake of only 0.1 gm. induced a distinctly positive nitrogen balance. To exclude the possibility that the apparent adequacy of this ration might be a transitory phenomenon, the test was continued for an additional period of 6 days. The result checked the previous finding, and showed definitely that 0.1 gm. of methionine, when accompanied by an abundant supply of L-cystine, was sufficient to meet the needs of this subject.

The observations described thus far naturally suggested the possibility that in man, in contrast to the rat, cystine might be capable of replacing methionine entirely. That this is *not* the case is disclosed by the results obtained during the last three periods of the experiment upon R. L. W. With the exclusion of methionine, while the administration of 0.81 gm. of L-cystine was continued daily, a slightly negative balance ensued. If one neglects the 1st day of the period, when the balance was still positive because of a carry over effect of the preceding diet, and calculates the average balance for the last 6 days only, a figure of −0.10 gm. is obtained. This is quite comparable in magnitude to the value of −0.15 gm. observed with the same ration during the penultimate period. The final period again showed the adequacy of a daily intake of 0.1 gm. of DL-methionine when cystine also was being consumed.

The effect of L-cystine, as outlined in the above experiment, greatly exceeded our expectations. The decrease in methionine requirement from 0.9 to 0.1 gm. daily represents a sparing action of approximately 89 per cent. Furthermore, the data demonstrate that cystine, though incapable of meeting the full methionine needs of man, actually diminishes the severity of the negative balances which follow the consumption of diets devoid of both these amino acids (*cf.* Rose *et al.* (10)). On the other hand, attention should be called to the fact that the subjective symptoms associated with methionine deprivation (10) are not prevented by the ingestion of L-cystine. More will be said about this in connection with the next experiment.

Finally, mention should be made of the very slight decrease in nitrogen intake during the last period (Table I). As will be seen later, this occurred even earlier during the second experiment. In both instances, it came

about through the use in the wafers of a new shipment of starch having a slightly lower nitrogen content. The differences are too small to be significant; indeed, such quantities are well within the limits of accuracy of the nitrogen balance technique.

Subject A. A. S. served in the second experiment. This young man was quite slender and undoubtedly would be regarded by most authorities as being under weight. He was 27 years of age and 5 feet 7 inches in height. When first placed on the diet, he weighed 58.8 kilos. The "ideal" weight of a male of this height at age 25 years is said to be 66.0 kilos (*cf.* Sherman (13)). It has been the experience in this laboratory that very thin individuals do not attain nitrogen balance readily upon diets containing mixtures of amino acids unless the energy intakes are raised above the usual figure of 55 calories per kilo. Reference was made to this fact in an earlier paper (14).

The ration which Subject A. A. S. first received furnished 55 calories per kilo. It was composed of amino acid Mixture 205 (11), Wafers V (described previously (12)), 190.2 gm. of extra sucrose, and 26.0 gm. of extra butter fat. After an adjustment period of 9 days, the subject was still in negative nitrogen balance, although his body weight had increased by about 1.0 kilo. At this point, his energy intake was raised to 58 calories per kilo, on the basis of his original weight, by the daily addition to the previous diet of 32.0 gm. of sucrose and 5.3 gm. of butter fat. No alteration was made in the amino acid mixture or wafers. The effect of this change in energy intake is shown by the figures representing the first period of the experiment as summarized in Table II. As will be observed, an average daily nitrogen balance of +0.04 gm. occurred. Since this was so slightly positive, the test was continued for the next period without dietary modification. An average retention of 0.20 gm. of nitrogen daily resulted. However, a fact which is not disclosed by the average is that on 2 of the 5 days of the period the subject was in slightly negative balance. An occurrence of this sort introduces uncertainty in the interpretation. To overcome this, the energy intake was further raised, beginning with the third period, to the unusual figure of 60 calories per kilo. This induced perfectly consistent results; consequently, the fuel value of the food was maintained at this level for the remainder of the experiment. Of the 10.03 gm. of nitrogen furnished by the final diet, 0.33 gm. was of an unknown nature. During the last four periods of the experiment, the use of the new shipment of starch, as mentioned above, reduced the unknown nitrogen to 0.30 gm.

Parenthetically, it is of interest to note that the highest energy intake of Subject A. A. S., if expressed in terms of the so called "ideal" or "normal" weight of 66.0 kilos for a young man of his age and height, amounted to

slightly in excess of 53 calories per kilo. No satisfactory explanation can be offered to account for the enhanced energy requirement of an unusually thin individual. Possibly it is associated with the greater body surface per unit of body weight, and the greater heat loss resulting therefrom. In

TABLE II

Sparing Effect of Cystine on Methionine Requirement of Man

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
			Subject A. A. S.			
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
7	59.8	10.03	9.29	0.70	+0.04	2.0 gm. DL-methionine
5	60.1	10.03	9.12	0.71	+0.20	2.0 " "
6	60.1	10.03	9.00	0.67	+0.36	2.0 " "
6	61.1	10.03	9.07	0.71	+0.25	1.0 " "
6	61.1	10.03	9.39	0.73	−0.09	0.9 " "
6	61.1	10.00†	9.11	0.73	+0.16	0.2 " " " 0.81 gm. L-cystine‡
4	61.0	10.00	9.70	0.61	−0.31§	No DL-methionine; 0.81 gm. L-cystine
4	60.5	10.00	9.24	0.73	+0.03	0.3 gm. DL-methionine; 0.81 gm. L-cystine
5	61.1	10.00	9.71	0.74	−0.45	0.1 gm. DL-methionine; 0.81 gm. L-cystine

* The initial diet contained amino acid Mixture 205 (11). During the first two periods, the subject received 58 calories per kilo of body weight; thereafter, the energy intake was increased to 60 calories per kilo.

† The slight decrease in nitrogen intake during this and subsequent periods was due to the use in the wafers of a new shipment of starch which had a slightly lower nitrogen content.

‡ Equivalent in sulfur content to 1.0 gm. of methionine.

§ Averages represent first 2 days only of the period. As a result of the deficiency, the subject experienced a severe loss of appetite and vomited part of the diet on the 3rd day. See the text.

any event, the phenomenon is one to be reckoned with in nitrogen balance studies, at least when the diets contain mixtures of amino acids in place of proteins.

As pointed out above, a consistently positive nitrogen balance occurred throughout the third period of the experiment upon A. A. S. (Table II). At that time, the methionine dosage was 2.0 gm. daily. During the next two periods, the intake of methionine was decreased, first to 1.0 gm. and

then to 0.9 gm. per day. The results indicate that 1.0 gm. daily was the smallest amount which was capable of maintaining a distinctly positive nitrogen balance. Therefore, this quantity represented the minimal requirement of Subject A. A. S. when cystine was absent from the food. The diet was then supplemented with 0.81 gm. of L-cystine daily, and, in view of the experience gained in the preceding experiment, the methionine dosage was dropped to 0.2 gm. daily. A distinctly positive balance resulted, with an average daily nitrogen retention of 0.16 gm.

During the next period, the *exclusion* from the food of the small quantity of methionine being ingested exerted a remarkable effect. On several occasions (*cf.* Rose *et al.* (10, 15)), the fact has been emphasized that depriving a human subject of one of the eight essential amino acids leads to a profound failure in appetite, a sensation of extreme fatigue, and a marked increase in nervous irritability. In the present experiment, these symptoms followed methionine deprivation even though cystine was being consumed. So marked was the appetite failure that A. A. S. found it necessary to force himself to eat the food. This he courageously contrived to do for 2 days, but, following breakfast on the 3rd day, part of the meal was lost by vomiting. During the remainder of the 3rd and all of the 4th day the subject was unable to ingest part of his ration. Obviously, this régime could no longer be maintained. The figures for the period (Line 7, Table II) represent the first 2 days only, during which all of the ration was consumed and retained. The average daily nitrogen loss is shown as 0.31 gm., but really was increasing quite rapidly and amounted to 0.51 gm. on the 2nd day.

To reestablish nitrogen equilibrium and restore the appetite of Subject A. A. S., 0.3 gm. of methionine was administered daily, along with 0.81 gm. of L-cystine, during the penultimate period. The effect was just as dramatic as that resulting from the exclusion of methionine. The average nitrogen retention for the period was only 0.03 gm., but the figures for the 4 days were −0.60, +0.07, +0.20, and +0.44 gm., respectively. Along with the change in nitrogen balance came a restoration of appetite, so that no further difficulty was experienced in consuming the ration until late in the final period, when a decrease in the methionine intake to 0.1 gm. daily occasioned a steadily increasing negative balance. The experiment was discontinued on the 5th day of lowered methionine consumption in order thereby to avoid the possibility of vomiting. The data demonstrate unequivocally that 0.2 gm. of methionine was the minimal daily requirement of the subject when cystine was present in the food. Thus, an abundance of cystine in the food reduced the minimal methionine needs of A. A. S. from 1.0 to 0.2 gm. daily. This represents a sparing action of approximately 80 per cent. On the contrary, in this subject, as in the preceding

experiment upon Subject R. L. W., L-cystine was incapable of replacing *all* of the methionine of the food.

TABLE III

Sparing Effect of Cystine on Methionine Requirement of Man

Period averages.

Period	Initial body weight	Daily N intake	Average daily N output		Average daily N balance	Diet notes*
			Urine	Feces		
			Subject A. G.			
days	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	
5	78.8	10.10	9.04	0.71	+0.35	0.6 gm. DL-methionine; 0.81 gm. L-cystine†
5	79.4	10.10	8.83	0.74	+0.53	0.5 gm. DL-methionine; 0.81 gm. L-cystine
6	79.4	10.10	9.02	0.69	+0.39	0.4 gm. DL-methionine; 0.81 gm. L-cystine
8	79.2	10.10	8.97	0.76	+0.37	0.3 gm. DL-methionine; 0.81 gm. L-cystine
8	79.8	10.10	9.06	0.73	+0.31	0.2 gm. DL-methionine; 0.81 gm. L-cystine
7	80.4	10.10	9.21	0.80	+0.09	0.1 gm. DL-methionine; 0.81 gm. L-cystine
8	81.3	10.10	9.46	0.77	−0.13	No DL-methionine; 0.81 gm. L-cystine
4	81.9	10.10	9.80	0.86	−0.56	" " no L-cystine‡
5	81.5	10.10	9.32	0.63	+0.15	0.1 gm. DL-methionine; 0.81 gm. L-cystine
5	82.6	10.10	9.08	0.77	+0.25	0.1 gm. DL-methionine; 0.81 gm. L-cystine

* In an earlier experiment, the minimal DL-methionine requirement of this subject had been shown to be 0.8 gm. daily when the ration was devoid of cystine. In the present test, the initial diet contained amino acid Mixture 205 (11), except for the indicated quantities of methionine and cystine.

† Equivalent in sulfur content to 1.0 gm. of methionine.

‡ During this period only, the subject received 1.22 gm. of L-tyrosine daily for the reason explained in the text.

The results of the third experiment are summarized in Table III and require very little discussion. Several weeks earlier, the minimal methionine requirement of this young man (Subject A. G.) had been shown to be 0.8 gm. daily when the diet was devoid of cystine. The experiment in Table III is concerned only with the effects of the latter amino acid. During the interval between the two tests, the subject was at his home for the Christmas vacation and had gained about 1 kilo in body weight. On the

basis of the initial weight given in Table III, his energy intake was slightly under 54 calories per kilo. His initial diet was composed of amino acid Mixture 205 (11), except for the indicated quantities of methionine and L-cystine, Wafers IV (*cf.* Rose *et al.* (12)), 255.3 gm. of extra sucrose, and 41.6 gm. of extra butter fat. The nitrogen intake from all sources amounted to 10.10 gm., of which 0.40 gm. was of an unknown nature.

The data in Table III demonstrate clearly that, in the presence of L-cystine, the minimal methionine requirement of Subject A. G. was 0.1 gm. daily. The figures also reveal that cystine, as in the other subjects, was incapable of replacing the entire methionine needs, and show further that the exclusion of both methionine and cystine (Line 8, Table III) greatly intensified the negative nitrogen balance. This latter observation was, of course, anticipated from the findings in our previous studies (10). Throughout, Subject A. G. was unaware of the character of the dietary alterations. During the period when both methionine and cystine were removed, finely powdered L-tyrosine was incorporated each day in the amino acid solution, just as the sulfur-containing amino acids were added during other periods. The limited solubility of tyrosine was sufficient to disguise its true nature. Despite this precaution against psychological influences, the deficiency symptoms were intense. The subject experienced a loss of appetite, complained of headache, nervousness, and insomnia, and threatened to discontinue the experiment. For these reasons, the period was shortened to 4 days. As always, the symptoms disappeared with the return of the missing amino acid to the diet.

It is evident from the above that the inclusion of L-cystine in the food diminished the minimal methionine requirement of A. G. from 0.8 to 0.1 gm. daily, or exerted a sparing action of approximately 87 per cent. Thus, in the three subjects, the replacement value of L-cystine varied from 80 to 89 per cent. This observation should prove to be of considerable importance from the nutritional point of view. In those areas of the world in which native diets are low in methionine, little consideration appears to have been given to the beneficial effects of cystine. In the light of our experience, such rations should be reevaluated in so far as they contain significant amounts of the latter amino acid.

SUMMARY

Experiments have been conducted in normal young men for the purpose of ascertaining the sparing effect of L-cystine upon their minimal methionine needs. To accomplish this, the methionine requirement of each subject was first established in the absence of dietary L-cystine. An excess of the latter amino acid was then incorporated in the food, and the methionine requirement was again determined. The results demonstrate

that L-cystine is capable of replacing 80 to 89 per cent of the minimal methionine needs of adult man, as measured by the maintenance of nitrogen equilibrium. Attention is called to the significance of this finding, particularly in those areas of the world in which methionine appears to be the limiting amino acid in the native diets.

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THE ISOLATION OF 5,6-DIHYDROCYTIDYLIC ACID FROM THE ACID-SOLUBLE FRACTION OF RAT LIVER SLICES*

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The recent report of Hurlbert *et al.* (1) describing the separation of several purine and pyrimidine ribonucleotides from the acid-soluble extracts of tissues by the use of a gradient elution technique provides a convenient means for determining the interrelationship of simple precursors, nucleotides, and polynucleotides in biological systems.

It was of interest to apply similar methods for the isolation of anionic compounds biologically derived from 4-C^{14}-cytidine in an attempt to provide information concerning the mechanism of cytidine incorporation into nucleic acid pyrimidines (2). This approach resulted in the isolation of a new pyrimidine nucleotide which appears to be identical with 5,6-dihydrocytidine-5′-phosphate.

EXPERIMENTAL

Tissue Extract—A 5 per cent trichloroacetic acid fraction was obtained during the routine isolation of ribonucleic acid (RNA) from the cellular fractions of rat liver slices incubated with 4-C^{14}-cytidine, as described previously (2). The TCA present in the acid-soluble fraction was removed by washing the solution with equal volumes of ethyl ether until the aqueous extract was pH 3. The extract was brought to pH 8 with 5 N NH_4OH.

Ion Exchange Chromatography—The alkaline solution of the tissue extract was transferred to a 20 cm. × 1.0 cm. Dowex 2 (formate form) anion exchange column. The gradient elution technique described by Hurlbert *et al.* (1) for ion exchange chromatography was used, except that a slow rate of mixing was employed to improve the resolving power of the system. It may be calculated (2) that a gradual, almost linear rise in the formic acid concentration results if a 2 liter mixer volume and 9 N formic acid in the reservoir are employed. Experimental application of these conditions re-

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sulted in the separation of three isomers of each ribonucleotide from a yeast nucleic acid hydrolysate.[1] Separation of the three isomers of cytidylic acid was facilitated by collecting 0.5 ml. fractions during the initial stages of elution. 4 ml. fractions were collected during the remainder of the separation. The spectrophotometric and isotopic assays employed were described previously (3).

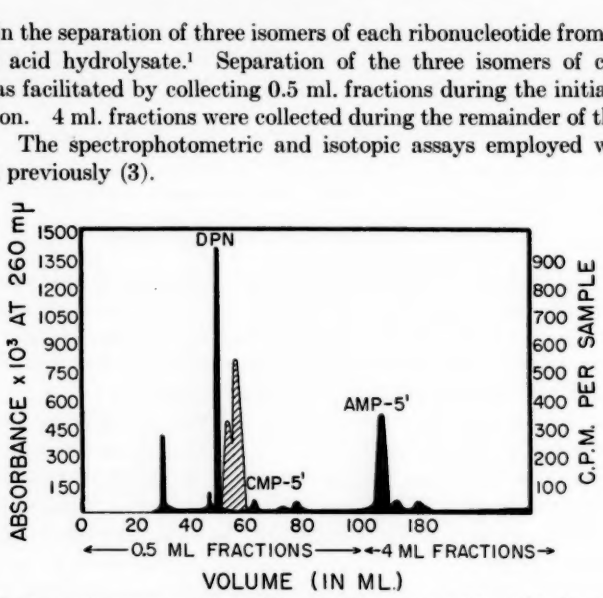

FIG. 1. Ion exchange separation of the trichloroacetic acid-soluble fraction of rat liver slices incubated with 4-C^{14}-cytidine (2). The solid peaks represent the ultraviolet absorption at 260 mμ, and the striped peaks represent the radioactivity in counts per minute per sample above background. This separation was accomplished with an anion exchange column (Dowex 1, formate form) 20.0 cm. in length and 1.0 cm. in diameter. The flow rate was maintained at 10 ml. per hour with a reservoir concentration of 9 N formic acid and a 2 liter mixer volume. 0.5 ml. fractions were collected during the initial stages of elution. After a total of 100 ml. had been collected, the sample volume was increased to 4.0 ml. DPN = diphosphopyridine nucleotide; CMP-5′ = cytidine-5′-phosphate; AMP-5′ = adenosine-5′-phosphate.

The results of the ion exchange separation of the anionic material present in the cytoplasmic acid solubles of rat liver slices incubated in the presence of 4-C^{14}-cytidine are given in Fig. 1. Larger quantities of the radioactive, non-ultraviolet-absorbing material desired for the identification of this component were obtained by pooling the acid-soluble extracts from ten incubations (5.0 gm. each) of rat liver slices with added non-labeled cytidine. One-fifth of the original radioactive peak was added to the large

[1] The yeast nucleic acid (hydrolyzed with snake venom diesterase) was kindly provided by Dr. A. Deutsch, California Foundation for Biochemical Research, Los Angeles, California. Appreciable amounts of the 2′- and 3′-nucleotides were present, because the incubation was carried out in the presence of suboptimal amounts of diesterase (pH 9.5) for a period of 2 weeks, resulting in alkaline hydrolysis during the enzymatic breakdown.

scale extracts for ion exchange separation. The conditions used for this separation are the same as those already described. The radioactive, non-ultraviolet-absorbing peak resulting from this separation was further purified by using the same mixer volume but a reduced acid concentration (2.0 N formic acid) in the reservoir. The results of this separation, given in Fig. 2, indicate that purification was effected satisfactorily.

Qualitative Identification—The selection of qualitative tests for determining the nature of the non-ultraviolet-absorbing compound was limited to

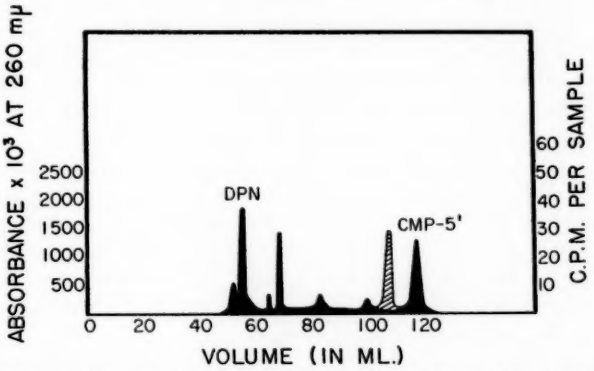

FIG. 2. Ion exchange purification of the unidentified non-ultraviolet-absorbing compound. The solid peaks represent the ultraviolet absorption at 260 mμ and the striped peaks represent the radioactivity in counts per minute per sample above background. This separation was effected on an anion exchange column (Dowex 1, formate form) 20.0 cm. in length and 1.0 cm. in diameter. The flow rate was maintained at 10 ml. per hour with a reservoir concentration of 2 N formic acid and a 2 liter mixer volume. 0.5 ml. fractions were collected. DPN = diphosphopyridine nucleotide; CMP-5′ = cytidine-5′-phosphate.

spot tests, since the total amount of the isolated material, from 50 gm. of rat liver slices, was approximately 1 mg.

Organically Bound Phosphorus—The methods described by Bandurski and Axelrod (4) and Hanes and Isherwood (5) were employed for organic phosphorus determinations.

Pentose Determination—The identity of the carbohydrate portion of the unknown was determined by the Brody cysteine (6) test for pentoses. Although a color is produced in the presence of either ribose or deoxyribose, a pure sample of either sugar may be easily characterized by the color produced. Ribose gives a pink to cherry-red color, and in the presence of deoxyribose a brown color develops. Similarly, uridylic acid or cytidylic acid produces a cherry-red color, whereas deoxycytidylic acid gives a brown color.

Nitrogenous Base Determination—The color test developed by Fink *et al.* (7) for the detection of carbamyl compounds and saturated pyrimidines was found to be specific and sensitive for the detection of as little as 5 γ of ureidosuccinic acid. Prior treatment with 0.1 N NaOH was required to obtain a positive test with dihydrouracil and dihydrothymine derivatives. Deamination of 5,6-dihydrocytosine derivatives with nitrous acid (5 per cent sodium nitrite in glacial acetic acid), however, was required prior to treatment with alkali to produce a yellow color in the presence of *p*-dimethylaminobenzaldehyde (Ehrlich's reagent).

Paper Chromatography—Two-dimensional descending paper chromatography of nucleotides was employed in which water-saturated phenol was used as the first solvent system and an equal mixture of secondary and tertiary butanol-saturated water as the second solvent system.[2] The spots were located according to the method of Bandurski and Axelrod (4).

Specific Activity Determinations—The undiluted radioactive material was purified by the ion exchange procedure described above, in which 2.0 N formic acid was used in the reservoir. The samples were plated on 1 inch watch crystals as described previously (3), and inorganic phosphorus was determined by the Werkheiser and Winzler (8) modification of the Fiske and Subbarow method (9). The specific activities were expressed as counts per minute per micromole of phosphorus.

Phosphate Ester Attachment—The location of the phosphate ester was determined enzymatically with crude snake venom.[3] The method of Werkheiser and Winzler (8) was employed for determining the release of inorganic phosphorus during enzymatic hydrolysis.

Results

The test for organic phosphorus applied to 25 μl. of the unknown sample (1 mg. in 1.8 ml. of water) resulted in a blue spot upon exposure to ultraviolet light. A 25 μl. sample of the unknown, in a spot plate, appeared red when heated in the presence of the pentose reagent. From these tests it was apparent that the unknown compound was a ribotide. The same volume of sample, however, did not produce a yellow color with *p*-dimethylaminobenzaldehyde directly after treatment with alkali. It seemed likely, nevertheless, that the unknown ribotide was structurally related to cytidylic acid, owing to the nature of the labeled substrate and its ion exchange behavior. Although an explanation is not readily apparent, it seems possible that an amino group on the 4 position of a dihydropyrimidine may

[2] The paper chromatography was kindly performed by Mr. C. McGaughey of the Veterans Hospital, Long Beach, California.

[3] The crude snake venom was generously supplied by Dr. A. Deutsch, California Foundation for Biochemical Research.

prevent hydrolysis to a carbamyl compound upon treatment with alkali. That this is indeed the case seems likely since deamination of a 25 μl. sample of either the unknown or hydrogenated cytidine-5′-phosphate,[4] followed by alkaline hydrolysis, resulted in characteristic color development in the presence of the reagent.

The R_F values of the unknown nucleotide obtained by two-dimensional paper chromatography were identical with those of the electrolytically reduced cytidine-5′-phosphate. Chromatography of the chemically reduced nucleotide and the biologically derived compound together resulted in a single spot having the same R_F values as the individual components. These two compounds had an R_F of 0.274 in the water-saturated butanol system and 0.254 in the second solvent system.

Incubation of a 25 μl. sample of the unknown nucleotide with crude snake venom monoesterase resulted in the release of approximately 1 γ of inorganic phosphorus. These results indicated that ribose was esterified at the 5 carbon. It was concluded from the above tests that the nucleotide biologically derived from cytidine is 5,6-dihydrocytidine-5′-phosphate.

Isotopic and phosphorus assays of the eluted samples derived after ion exchange purification of the undiluted radioactive material revealed that there was a constant specific activity of 1.2×10^4 c.p.m. per μmole of phosphorus through the entire peak. As a further indication of purity, the specific activity of the 5,6-dihydrocytidylic acid was almost the same as that of the original 4-C^{14}-cytidine.

DISCUSSION

The participation of dihydropyrimidines in pyrimidine metabolism has gained prominence from the results of the studies of Lieberman and Kornberg (10), Cooper and Wilson (11), and Fink and McGaughey (12). The latter workers, studying uracil and thymine catabolism, proposed a degradative mechanism for free pyrimidines involving an initial reduction followed by ring opening to form carbamyl derivatives. Lieberman and Kornberg (10) have shown that a similar mechanism is involved in pyrimidine synthesis; namely, conversion of ureidosuccinic acid to dihydroorotic acid followed by desaturation to orotic acid. Although Hurlbert and Potter (13) have shown that labeled orotic acid, in turn, is rapidly incorporated into uridine-5′-phosphate and several derivatives of this compound, there is conflicting evidence as to whether or not orotic acid is an obligate intermediate in *de novo* synthesis of pyrimidine nucleotides and polynucleotides (13–15).

The isolation of 5,6-dihydrocytidine-5′-phosphate does not imply that

[4] This compound was prepared by electrolytic reduction of cytidine-5′-phosphate; Fukuhara, T. K., and Visser, D. W., unpublished data.

the dihydronucleotide is an obligate intermediate of RNA pyrimidine synthesis, particularly since the specific activity of the nucleotide was the same as the 4-C^{14}-cytidine from which it was derived. On the other hand, the possibility that dihydronucleotides are intermediates of cytidine incorporation cannot be excluded on this basis since conclusions from dilution experiments in systems *in vitro*, such as the conditions used in these experiments, are rather tenuous. The observed transformation does show, however, that hydrogenation of pyrimidine nucleotides or nucleosides can occur and may be the manner in which cytidine is incorporated into normal acid-soluble precursors of polynucleotide pyrimidines.

A speculation that dihydropyrimidine nucleotides are normal intermediates would be in agreement with the evidence reported by Mitchell and Houlahan (14) that an aliphatic ribosyl compound is formed prior to pyrimidine ring formation if it is assumed that ring closure of an aliphatic ribotide is similar to the known mechanisms for pyrimidine ring formation from ureidosuccinate (10). Such a mechanism would also explain the finding of Hurlbert and Potter (13) that labeled orotic acid is not diluted *in vivo* as would be expected if a pool of orotic acid were present.

The amount of cytidine-5′-phosphate isolated from the acid-soluble fraction was too small for an accurate determination of its specific activity. There was, however, a detectable amount of radioactivity associated with this nucleotide. It would be expected that a direct conversion of labeled cytidine to cytidine-5′-phosphate would result in a decrease in specific activity, since cytidylic acid is present in the acid-soluble fraction whether or not cytidine is administered as a substrate. For reasons mentioned earlier, however, the fact that there is little or no dilution of isotope during the conversion of labeled cytidine to the dihydronucleotide does not negate the possibility that phosphorylation precedes hydrogenation of cytidine. That pyrimidine nucleotides are formed in liver tissue from the corresponding nucleoside has been shown by Browerman and Chargaff (16).

The conversion of cytidine to dihydrocytidylic acid in liver tissue poses some interesting questions. Are the dihydropyrimidine nucleotides involved in the *de novo* synthesis of pyrimidines or in pyrimidine breakdown, or is dihydrocytidylic merely an artifact resulting from the presence of an abnormal metabolite?

SUMMARY

Ion exchange methods including a gradient elution technique were modified for the analysis of the acid-soluble components of rat liver slices. Application of these methods resulted in the isolation of a hitherto unknown nucleotide biologically derived from 4-C^{14}-cytidine. Through the use of qualitative color tests and paper chromatography, this nucleotide was

identified as 5,6-dihydrocytidine-5′-phosphate. The possible significance of saturated pyrimidine nucleotides in nucleic acid biosynthesis is discussed.

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THE TRANSGLYCOSYLATIVE ACTION OF TESTICULAR HYALURONIDASE*

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Recently, there was reported the resolution of digests produced from hyaluronic acid by testicular hyaluronidase into a series of oligosaccharides (2). The sizes of the oligosaccharides isolated ranged from di- through tetradecasaccharide in regular increments of 1 disaccharide unit. As in hyaluronic acid itself (3), equal numbers of glucuronic acid and acetylglucosamine residues are linked in these oligosaccharides in an alternating structure. The free reducing group is on the terminal acetylglucosamine residue. The oligosaccharides represent well defined substrate models of hyaluronic acid, and availability of methods for their individual estimation in mixtures permits a more precise analysis of the mode of action of some mucolytic enzymes than was previously possible. This report is concerned with the application of these substrates and these methods to studies on the action of bovine testicular hyaluronidase.

EXPERIMENTAL

Methods and Materials—The qualitative and quantitative paper and ion exchange chromatographic procedures used (2), the isolation and properties of the oligosaccharide substrates (2), the preparation of hyaluronic acid and of a solid oligosaccharide mixture (4), and the conditions used in the Macleod and Robison hypoiodite reducing sugar method (2) have been described previously. The enzyme used in this study, except when otherwise stated, was a bovine testicular hyaluronidase prepared by an alcohol fractionation method, lot No. W-179, kindly supplied by Dr. Joseph Seifter of Wyeth, Inc. It assayed (5) about 1200 turbidity reducing units (abbreviated elsewhere as t.r.u.) per mg. With exceptions as indicated, all incubations were performed at 37° under toluene at a substrate concentration of 20 mg. per ml. in 0.1 M acetate buffer of pH 5, 0.15 M in sodium chloride, containing 1 mg. per ml. of gelatin. Where specified, cysteine hydrochloride and sodium pyrophosphate, both at 0.01 M concentration, were added.

* This work was reported in part before the meeting of the American Society of Biological Chemists at Chicago (1). It was supported by grants to Dr. Karl Meyer from the National Institutes of Health and the Helen Hay Whitney Foundation.

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XUM

The oligosaccharides are conveniently designated by roman numerals (2) representing their degree of polymerization, n, in the formulation GA-(AcGN-GA)$_{n-1}$-AcGN, where GA is a glucuronic acid residue and AcGN is an acetylglucosamine residue. Disaccharide is thus oligosaccharide I, tetrasaccharide is oligosaccharide II, etc.

Hyaluronic Acid and Testicular Hyaluronidase—Sodium hyaluronate was incubated with 600 t.r.u. per ml. of enzyme, and at 24, 72, and 144 hours fresh enzyme equivalent in amount to that initially present was added. At various times the oligosaccharide composition of the digest was determined by ion exchange columns and the reducing value was measured by

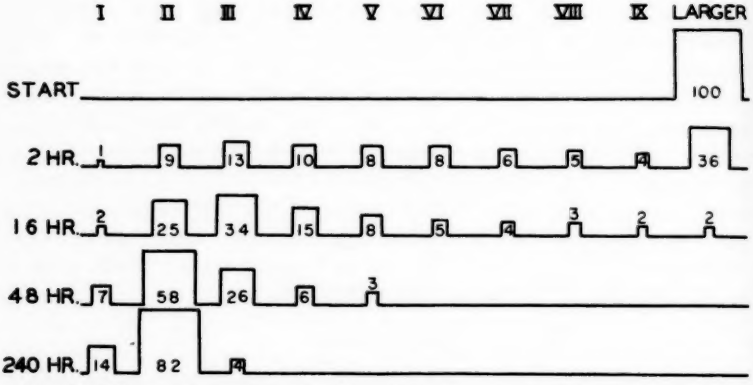

FIG. 1. Column chromatograms, schematically represented, showing the amounts of oligosaccharides I through IX in a testicular hyaluronidase digest of hyaluronic acid at various time intervals. The numerical values indicate the per cent of each product present. For the corresponding reducing sugar curve, see Curve A of Fig. 2.

the hypoiodite method. As shown in Fig. 1, the oligosaccharides formed display a random distribution early in the action, followed by their accumulation first at the hexasaccharide level, later at the tetrasaccharide level. The rate of liberation of end-groups in this experiment, and in a similar one conducted at 40 t.r.u. per ml., is presented in Fig. 2. Since only glucosaminidic linkages are split by the enzyme (2, 6), "per cent hydrolysis" is taken as the proportion of these linkages split, based on the earlier established value of 3.4 molecules of hypoiodite per acetylglucosamine end-group liberated (2). The hypoiodite consumption of the sodium hyaluronate used indicated a minimal molecular weight of 36,000 (90 disaccharide units), assuming this same end-group.

Isolated Oligosaccharides and Testicular Hyaluronidase—Oligosaccharide III (hexasaccharide, Preparation E-III) was incubated with 600 t.r.u. per

ml. of enzyme; 1200 t.r.u. per ml. of fresh enzyme were added at 24 and at 48 hours. Results of quantitative paper chromatography of the digest at various times are shown in Fig. 3. It is seen that in the initial action not only *lower* oligosaccharides are produced, as expected, but also *higher* oligosaccharides. After prolonged digestion, the products were similar in quantity to those produced from hyaluronic acid.

The oligosaccharides from tetrasaccharide through dodecasaccharide gave qualitatively similar results. The extremely wide variation in rate of enzyme action through this series necessitated adjustment of enzyme

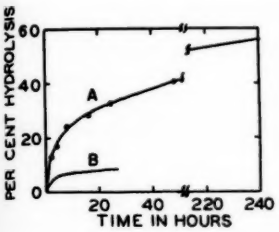

FIG. 2

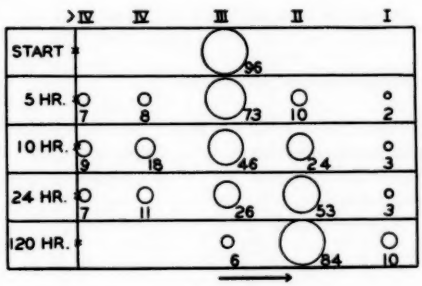

FIG. 3

FIG. 2. Liberation of reducing groups from hyaluronic acid by testicular hyaluronidase, measured by a hypoiodite method, expressed as per cent hydrolysis to disaccharide. Curve A, 600 t.r.u. per ml., with fresh enzyme additions at 24, 72, and 144 hours. Curve B, 40 t.r.u. per ml.

FIG. 3. Paper chromatograms, schematically represented, showing the action of testicular hyaluronidase on the hexasaccharide. The roman numerals at the top designate the oligosaccharides formed. The numerical values indicate the per cent of each product present, determined by elution and colorimetry.

level and time in order to demonstrate the primary products of action from each substrate, as shown in Table I. The initial appearance in each case of higher as well as of lower members of the series is noted. Limited experiments with hexasaccharide and octasaccharide showed roughly a linear relationship between initial rate of action and enzyme concentration or time. Extending this to the other oligosaccharides, it is possible to compare the approximate relative rates of initial action, expressed as micrograms of substrate consumed per hour per turbidity reducing unit of enzyme. These rates appear in the last line of Table I.

In Table II results are summarized for digestion of each substrate for 240 hours at 600 t.r.u. per ml., with additions of 600 t.r.u. per ml. of fresh enzyme at 48, 96, 144, and 192 hours. It is seen that oligosaccharides II through VI all yield the same products of prolonged digestion as hyaluronic

acid (Fig. 1) under like conditions, and that the proportion of disaccharide produced can be varied by changing the time or substrate concentration.

To determine whether the disaccharide, which itself undergoes no change with the enzyme, was an intermediate in the production of higher oligosaccharides, the hexasaccharide was subjected to prolonged digestion in the

TABLE I

Initial Products of Testicular Hyaluronidase Action on Individual Isolated Oligosaccharides

Digestion conditions and products	Substrate, oligosaccharide No.				
	II	III	IV	V	VI
Oligosaccharide preparation No.	R-II	E-III	R-IV	R-V	R-VI
Enzyme level, *t.r.u. per ml.*	600*	600	40†	40†	40†
Digestion time, *hrs.*	240	8	8	2	2
Method of analysis	Column	Paper	Column	Column	Column
Products, %					
Oligosaccharide I	10	1	1	1	1
" II	88	15	5	2	6
" III	2‡	65	4	8	7
" IV		13	69	2	8
" V			13	67	6
" VI			15	4	23
" VII			2	9	7
" VIII			1	4	3
Unresolved or larger oligosaccharides		6	1	3	39§
Relative rate of action‖	0.01	1.4	20	82	190

* At 48, 96, 144, and 192 hours, 600 t.r.u. per ml. of fresh enzyme were added.

† Buffer contained cysteine and pyrophosphate.

‡ Identity checked by paper chromatography; not present in substrate.

§ This figure, representing material not recovered from the column and computed by difference, is considerably less reliable than the other values.

‖ Micrograms of substrate consumed per hour per turbidity reducing unit of enzyme.

presence of the disaccharide. The products, after subtraction of disaccharide initially added, were not changed (Table II).

Effect of pH—Octasaccharide (Preparation P-IV) was incubated for 2 hours with 600 t.r.u. per ml. of enzyme, the acetate buffer being replaced by 0.1 M phosphate-citrate buffers containing sodium chloride and gelatin. Qualitative paper chromatography showed that the action observed at pH 4.2 or 4.7 was similar to that observed with acetate buffer at pH 5, excluding the participation of inorganic phosphate or of phosphorylated

intermediates in the action. The action at higher pH was similar in pattern, but perceptibly less pronounced at pH 5.4 or 6.2 and considerably less at pH 6.9. Similar pH dependency has been found for early stages of the action of the enzyme on hyaluronic acid as measured by reducing sugar methods (7).

Action of Various Bovine Hyaluronidase Preparations—The product patterns obtained by digestion of octasaccharide (Preparation P-IV) with three different enzyme preparations, all used at a level of 50 t.r.u. per ml., were compared by quantitative paper chromatography. The enzymes were (*a*) Preparation M-IV-43-C-II, obtained by lead acetate fractionation (8) assaying 800 t.r.u. per mg.; (*b*) Preparation W-179, the alcohol-fractionated commercial preparation used elsewhere in this work; (*c*) Prep-

TABLE II

Products of Prolonged Digestion of Oligosaccharides at High Enzyme Levels

The figures are expressed in per cent.

Products	Substrate, oligosaccharide No.							
	I	II	III	III*	III†	IV	V	VI
Oligosaccharide I	100	10	13	20	13	11	13	11
" II		88	85	72	84	88	85	87
" III		2	2	8	3	1	2	2

* Substrate concentration, 80 mg. per ml.; all others, 20 mg. per ml.

† 4 mg. per ml. of disaccharide added at start of digestion; this amount was subtracted from the products.

aration W-179-H, prepared by heating the second preparation, dissolved at 0.5 mg. per ml. in the usual buffer, for 90 seconds (in a narrow test-tube) in a boiling water bath and chilling at once. This last preparation, a turbid solution, was stored for 5 hours at 4° before start of the digestion, during which time it was assayed. It had 7 per cent of the activity of the unheated enzyme. The results with these preparations are summarized in Table III. The extent of action and product patterns of the first two preparations are in satisfactory agreement. The third, heated preparation gives a similar product pattern, but at an accelerated rate; the pattern after 4 hours with the heated enzyme is comparable with that observed after 16 hours with the other two. This discrepancy is only apparent, as has become clear from recent findings (9, 10) which show that heat-inactivated testicular hyaluronidase preparations regain a substantial portion of their original activity on standing.

Absence of Enzyme Action on Reduced Hexasaccharide—Solution of 10

mg. of the hexasaccharide (Preparation R-III) in 0.1 ml. of 0.1 M sodium borohydride solution in 0.05 M sodium bicarbonate was followed after 10 minutes by acidification to pH 5 with acetic acid. The resulting reduced hexasaccharide preparation and other members of the oligosaccharide series similarly reduced moved slightly more slowly on paper than the corresponding members of the series before reduction. Both series were readily revealed on paper by exposure to chlorine, followed by spraying with starch-iodide solution (11). The expected complete reduction (12) of the reducing acetylglucosamine end-groups to acetylaminoglycitol end-groups was demonstrated by failure to develop color on paper, in contradistinction

TABLE III

Comparison of Action on Octasaccharide of Various Testicular Hyaluronidase Preparations, Used at Same Level of Activity

The figures are expressed in per cent.

Products	Enzyme used*					
	None (control)	M-IV-43-C-II		W-179		W-179-H	
		4 hrs.	16 hrs.	4 hrs.	16 hrs.	4 hrs.	16 hrs.
Oligosaccharide II		2	13	6	16	14	25
" III	1	5	23	7	25	20	35
" IV	97	81	35	73	31	39	21
Larger than oligosaccharide IV	2	12	29	14	28	27	19

* For the description of the enzyme preparations see the text. The digestion times are as indicated.

to the starting materials (2), with acetylacetone and dimethylaminobenzaldehyde.

The reduced hexasaccharide, incubated for 48 hours with enzyme at 250 t.r.u. per ml. in the presence of cysteine and pyrophosphate, was unchanged as shown by paper chromatography and chlorine treatment. Unreduced hexasaccharide, incubated as a control in the presence of acidified borohydride solution, underwent the usual type of action.

Inhibition Effects—Oligosaccharides obtained directly from columns of Dowex 1 were purer by analysis for hexosamine and uronic acid than the preparations from paper chromatography and alcohol fractionation (2) used at first (1). Unlike these, however, they were not acted upon by the enzyme. The subsequent Amberlite IRC-50 treatment (2) demonstrably removed Cu^{++}. The resulting substrates still showed strong inhibition in the usual buffer, but on addition of cysteine and pyrophosphate their

reactivity was equal to or comparable with that of the paper preparations. Cysteine and pyrophosphate, as complex-forming agents effective in reversing inhibition by Cu^{++} and Fe^{+++} of the enzymatic action on hyaluronic acid (13), are apparently most efficient with the higher molecular weight substrates (Table IV). When the rate of action was decreased by inhibition effects, the product pattern remained the same.

Action of the enzyme on hyaluronic acid at 2 mg. per ml., as measured by the turbidimetric method (5), was inhibited to the extent of 35 per cent on adding 10 mg. per ml. of a mixture of oligosaccharides (oligosaccharide II, 25; III, 33; IV, 24; and higher, 19 per cent). Oligosaccharide II alone produced no inhibition.

Other Hyaluronidases—The possible production of higher oligosaccharides from hexasaccharide or octasaccharide by some hyaluronidases other than testicular hyaluronidase was briefly investigated by qualitative paper chromatography. A snake venom hyaluronidase (*Crotalus terrificus*, dried venom, assaying 75 t.r.u. per mg.) (14), incubated under the conditions of Table I with these two oligosaccharides, gave patterns similar to those produced by the testicular enzyme, *i.e.* formation of products with the mobilities of both higher and lower members of the oligosaccharide series. No disaccharide was detected. An extract of leech heads (14, 15) was prepared by water extraction, precipitation with 0.75 saturated ammonium sulfate, dialysis, and lyophilization (assay, 53 t.r.u. per mg.). From hexasaccharide this enzyme gave products with the mobilities on paper of tetrasaccharide and disaccharide, but no higher members of the series were detected. The action of pneumococcal hyaluronidase on oligosaccharides is described elsewhere. This enzyme does not produce higher molecular weight products from oligosaccharide substrates, but its hydrolytic action is quite complex (16).

TABLE IV

Inhibition of Hyaluronidase Action by Substrate Impurities

Method of preparation of substrates	Relative rate of action on oligosaccharides, γ substrate consumed per hr. per t.r.u. enzyme			
	III	IV	V	VI
Alcohol fractionation	1.4			
Paper chromatography		33	100	190
Ion exchange chromatography	<0.1	<2		
Purified ion exchange preparation	0.4	~5	<10	<20
" " " " with cysteine and pyrophosphate	0.7	20	82	190

DISCUSSION

Two simultaneous types of action are observed with the enzyme preparation. One is the expected hydrolytic cleavage of glucosaminidic linkages in the oligosaccharides, which increases the number of free reducing groups (and molecules) in digests, resulting eventually in production of low molecular weight oligosaccharides (2, 6). A second type of enzymatic action produces higher oligosaccharides. In the absence of energy sources in the digests, it is clear that the apparent synthesis must be a transglycosylative process, involving only minor changes in free energy. Other examples among the carbohydrases are now well known. In the present case, fragments containing multiples of 1 disaccharide unit are transferred from 1

TABLE V

Initial Action of Testicular Hyaluronidase on Isolated Oligosaccharides Computed on Molar Basis

Substrate	Moles of oligosaccharides produced per mole of substrate consumed									
	I	II	III	IV	V	VI	VII	VIII	IX	Total
Oligosaccharide III..	0.10	0.64		0.28	0.10*					1.12
" IV..	0.13	0.32	0.17		0.32	0.32	0.04	0.02	0.02*	1.34
" V...	0.08	0.15	0.43	0.08		0.10	0.19	0.08	0.05*	1.16
" VI..	0.08	0.23	0.18	0.16	0.09		0.08	0.03	†	†

* Because of incomplete resolution of higher molecular weight material, these values may include some products of larger size than represented.

† Oligosaccharide IX and larger products, not recovered from the columns and calculated by difference, amounted to 0.34 mole, based on the molecular weight of oligosaccharide IX. The products thus totaled less than 1.19 moles.

substrate molecule to another, with the breaking of one glucosaminidic linkage and the concomitant formation of a new one.

Analysis of the initial action (Table I) is facilitated by computing the number of moles of each product appearing for the disappearance of 1 mole of substrate (Table V). In the four cases considered it is seen that the total amount of product ranges from 1.1 to 1.3 moles for various oligosaccharides. The net increases, 0.1 to 0.3 mole, are measures of hydrolysis. While these values are only approximate because of additive errors, they show that hydrolysis can represent only a small part of the action occurring and that the larger part of the substrates is consumed in transglycosylation.

The types of action necessary to account for the products initially formed from oligosaccharides are best exemplified by attempting an analysis of the action on hexasaccharide. Summation of the accompanying equations, in which the numerical values are moles of oligosaccharides,

reproduces the over-all action (Table V). For simplicity, only five reactions have been selected from among the many possible.

$$0.80\ \text{III} \rightarrow 0.40\ \text{II} + 0.40\ \text{IV}$$

$$0.08\ \text{III} + 0.08\ \text{IV} \rightarrow 0.08\ \text{II} + 0.08\ \text{V}$$

$$0.04\ \text{III} \rightarrow 0.02\ \text{I} + 0.02\ \text{V}$$

$$0.08\ \text{III} + 0.08H_2O \rightarrow 0.08\ \text{I} + 0.08\ \text{II}$$

$$0.04\ \text{IV} + 0.04H_2O \rightarrow 0.08\ \text{II}$$

$$1.00\ \text{III} + 0.12H_2O \rightarrow 0.10\ \text{I} + 0.64\ \text{II} + 0.28\ \text{IV} + 0.10\ \text{V}$$

The selection of these reactions is partly arbitrary. It is clear, however, that the first equation, meeting uniquely the requirement of a transfer process not producing oligosaccharide I, must be the most important. The second equation, accounting for the production of oligosaccharide V and, in part, for the large excess of oligosaccharide II over oligosaccharide IV, has been chosen from considerations of enzyme specificity discussed later. There are reasons to believe that the last three equations, and undoubtedly others not represented, also contribute in a minor part to the over-all action. While analysis of the initial action on other oligosaccharides is even more complex, the nature of the products shows that analogous processes occur here. Similar action patterns must prevail in hyaluronidase digests of hyaluronic acid, except that far greater interaction between different species would be expected.

While definitive proof can only come from rigorous enzyme purification, present evidence points strongly to catalysis of hydrolysis and transglycosylation by a single enzyme: (*a*) There is no change in product pattern when the enzyme is 93 per cent inactivated by heating or when an enzyme prepared by a different method is substituted. (*b*) The relative proportion of hydrolysis to transglycosylation is similar for oligosaccharides III through VI, although the over-all reactivity changes about 100-fold; *i.e.*, specificity varies with substrate in the same way for both actions. (*c*) No change in action pattern is caused by inhibitors or by varying the pH from 4 to 7. (*d*) Reduction of the free end-group of the hexasaccharide abolishes both types of action. (*e*) Catalysis, as here, of both transglycosylation and hydrolysis by one enzyme has been demonstrated for a transgalactosylase (17) and some sucrases (18) and may be quite common.

With the same reservation, identity of the enzyme acting on oligosaccharides with that degrading hyaluronic acid is shown by the following evidence: (*a*) The two different enzymes and the heat-inactivated preparation have comparable relative activity for the polymer and the oligosaccharides. (*b*) The pH dependency of the two actions is similar. (*c*) Ac-

tion on the oligosaccharides and the polymer gives similar products under similar conditions. (*d*) The inhibition by oligosaccharides of the action on hyaluronic acid, the apparent inhibition by Cu^{++} of action on the oligosaccharides, and the activation by cysteine are suggestive. (*e*) The shape of reducing sugar curves for enzyme action on the polymer is satisfactorily explained by use of data obtained with the oligosaccharides, as will be shown.

At widely different enzyme levels, the net hydrolysis of hyaluronic acid exhibits an initially rapid rate, followed by what appears as an approach to a limiting value (Fig. 2). Curves of similar shape are obtained at levels as low as 3 t.r.u. per ml. (7). This rapid falling off of the rate of hydrolysis, despite the presence of substrate which can still be split by the enzyme, is consistent with an extrapolation to high molecular weights of the decrease in rate of action with decrease in molecular weight which is observed with the oligosaccharides. The accumulation in late stages of the action (Fig. 1) of fragments first at the hexasaccharide level and later at the tetrasaccharide level reflects the large differences in reactivity between oligosaccharides IV and III and between oligosaccharides III and II (Table I). The smaller differences in reactivity higher in the series explain the apparently random distribution of products earlier in the action. It appears likely that transglycosylation occurs with hyaluronic acid, as with the oligosaccharides. What biological significance this may have, if any, is not now clear. The action as above, at a much slower rate, of a polysaccharase on oligosaccharides which it produces from a polymer is encountered elsewhere (19, 20).

Present evidence indicates that the enzyme forms only one type of glucosaminidic linkage, the same as that present in hyaluronic acid: (*a*) Paper or column chromatography fails to demonstrate substantial heterogeneity of oligosaccharide II; since this substance is the principal product of action on oligosaccharide III, it must contain linkages formed by the enzyme. (*b*) The rapidity of transglycosylation relative to hydrolysis implies that the enzyme must split the linkages which it forms. (*c*) Hyaluronidases from leech heads, snake venom, and the pneumococcus act on both hyaluronic acid and the testicular hyaluronidase oligosaccharides. The homogeneity of bond type in digests here indicated is analogous to that recently found in the disproportionation of amylose oligosaccharides by D-enzyme (21).

Of considerable interest is the initial production from oligosaccharide V of more oligosaccharide III than IV; analogous alternation of products is observed with oligosaccharide IV or VI as substrate (Table V). Preferential attack by the enzyme on linkages at least 2 disaccharide units removed from either end would be consistent with this behavior. Such a preferential specificity requirement would also explain the slow formation

of disaccharide, as well as the sharp drop in reactivity between oligosaccharides IV and III and between oligosaccharides III and II. Suggested as consistent with the observations is a periodicity of binding sites on the enzyme surface which corresponds to the known periodicity of the substrate structure. Binding sites on either side of the linkage attacked would exert an orienting effect, bringing the reacting centers of enzyme and substrate into close proximity. Conceivably, slightly wider spacing of the binding sites of the enzyme than those of the substrate would likewise increase susceptibility of interior linkages to attack. In either case, affinity of the enzyme for a substrate would increase with the number of binding sites offered by the latter. With regard to the process occurring at an active center of the enzyme (not necessarily different from a binding site), the most likely one appears to be exchange of a carbohydrate-enzyme linkage for the original carbohydrate-carbohydrate linkage (22). One fragment of the substrate thus cut off eventually leaves the enzyme. The linkage between the enzyme and the remaining fragment may be hydrolyzed. More frequently, it is exchanged for a new carbohydrate-carbohydrate linkage, joining the second fragment with a cosubstrate molecule which has replaced the first fragment on the enzyme surface.

SUMMARY

The individual oligosaccharides isolated earlier from testicular hyaluronidase digests of hyaluronic acid show two types of reaction when acted upon by this enzyme. Fragments containing multiples of 1 disaccharide unit are transferred rapidly from one substrate molecule to another (transglycosylation). A slower simultaneous hydrolysis leads eventually to products of low molecular weight. The rate of action increases markedly with substrate size. Evidence presented indicates that the same enzyme catalyzes both types of action on the oligosaccharides, and likewise on hyaluronic acid. The findings are applied to an analysis of the action on hyaluronic acid, and a hypothesis which relates features of the enzyme action to substrate structure is considered. Comparisons with other carbohydrases are made.

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TRACER STUDIES OF THE ACIDS OF THE TRICARBOXYLIC ACID CYCLE

I. THE FATE OF LABELED ACETATE IN THE LIVERS OF NORMAL AND DIABETIC RATS*

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Recent work has suggested that a block exists between pyruvate and the tricarboxylic acid cycle in the alloxan-diabetic rat (1, 2). Further studies concerning the entrance of labeled key intermediates into the cycle in normal and diabetic animals would be of value in furthering knowledge of the chemical lesion in alloxanized rats as well as elucidating the pattern of the metabolic disposal of these intermediates by normal animals. The development of a fluorometric method for the quantitative determination of the acids of the tricarboxylic cycle following chromatography has facilitated measurement of the concentration of these acids in tissues (3). With this new procedure accompanied by parallel tracer studies, the fate of labeled acetate in normal and diabetic rats was investigated.

Labeled acetate was chosen as the first intermediate for study because of its ready availability and because of the large mass of data concerning its metabolic fate. Liver was the tissue of choice, since, with kidney, its metabolic patterns appear to be altered more in alloxan diabetes than those of other tissues (1). Furthermore, the acids formed in liver may be extracted without contamination by the excretory products which complicate the interpretation of results obtained from kidney.

EXPERIMENTAL

Male albino rats of the Sprague-Dawley strain weighing 200 to 300 gm. were used throughout the investigation. The rats made diabetic were treated with alloxan by a method described previously (1); only those animals showing blood sugar values greater than 250 mg. per cent were used. For at least 2 weeks prior to the experiment, all animals were fed a diet consisting of 20 per cent casein, 30 per cent sucrose, 41 per cent glucose, 5 per cent cottonseed oil, 4 per cent salt mixture (Wesson's), and an adequate vitamin mixture. After the animals had been fasted for

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18 hours, each was given intracardially 0.025 mc. of acetate-1-C^{14} (approximately 0.5 to 2.5 mg. of sodium acetate). At 5 and 15 minute intervals following injection, groups of animals were sacrificed and their livers were removed and treated as described in detail previously (3), the livers of five rats being pooled for each set of duplicate determinations. Tissues from three separate sets of normal and of alloxanized rats were analyzed

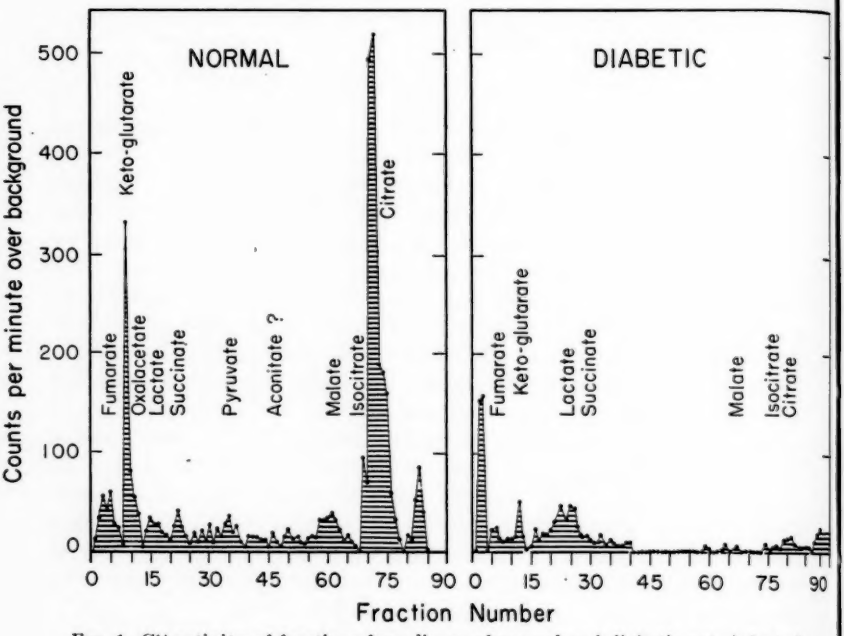

FIG. 1. C^{14} activity of fractions from livers of normal and diabetic rats injected with labeled acetate.

after both the 5 and the 15 minute periods. The tissues were frozen, homogenized in acetone, and extracted with acid-acetone, after which the extracted acids were chromatographed on hydrated silica gel and determined fluorometrically (3). Samples from two different pools of livers from both normal and diabetic rats were chromatographed and the fractions thus obtained were evaporated and counted in a proportional gas flow counter in order to determine the activity of the various acids. Typical chromatograms in which fraction number is plotted against counts per minute per fraction of both diabetic and normal livers are shown in Fig. 1.

The concentrations and counts per millimole of the acids of the cycle in normal rat livers 5 and 15 minutes after injection with acetate-1-C^{14} are shown in Table I. Since the activity is much higher 5 minutes after injection, the peak in counts per millimole of acid must occur very soon after

TABLE I

Levels and C^{14} Activities of Acids in Livers of Normal Rats after Injection with Acetate-1-C^{14}

Acid	Level, mg. per 100 gm. tissue		C.p.m. per mmole	
	5 min. after	15 min. after	5 min. after	15 min. after
Citrate	2.9	2.4	2156	128
Aconitate	0.4	0.5	183	101
Isocitrate	0.7	0.7	336	113
α-Ketoglutarate	1.4	1.7	959	96
Succinate	3.0	2.4	74	28
Fumarate	1.7	1.8	157	49
Malate	1.7	1.8	255	24

TABLE II

Levels and C^{14} Activities of Fractions from Livers of Normal and Diabetic Rats after Administration of C^{14}-Acetate

Fraction	Level, mg. per 100 gm. tissue		C.p.m. per 100 gm. tissue		C.p.m. per mmole × 10^{-3}	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
Citrate	2.9	1.0	32,600	500	2156	96
Aconitate	0.4	*	420	*	183	*
Isocitrate	0.7	0.2	4,560	100	336	96
α-Ketoglutarate	1.4	0.5	7,800	160	959	29
Succinate	3.0	1.0	1,880	140	74	21
Fumarate	1.7	0.6	2,300	1200	157	139
Malate	1.7	0.7	3,240	140	255	23
Chloroform-mobile fraction (fat, etc.)			4,680	7540		

* Too low for accurate measurement.

administration of the labeled compound. This is in agreement with the work of Marshall and Friedberg (4). Owing to the much greater specific activity of the cycle acids 5 minutes after injection, this period was chosen for subsequent studies in the diabetic animals. After a shorter period the acids might show even higher specific activities, but such short intervals would be technically inconvenient.

Table II shows the levels and activities of the acids in the livers of normal and diabetic rats 5 minutes after injection with acetate. Both counts per millimole and counts per 100 gm. of tissue are given. It is evident that both the levels of the cycle acids and their C^{14} activities are much lower in the alloxanized animals than in the normal. Owing to the low levels of some of the acids in the diabetic animals, the fractions may be expressed more precisely as counts per 100 gm. of tissue than as counts per millimole.

DISCUSSION

In the acids of the cycle, both the counts per 100 gm. of tissue and the counts per millimole of acid were lowered in the diabetic liver (Table II). This indicates that acetate was not entering the cycle at the same speed as in the normal controls. This is not due merely to a general decrease in the rate of metabolic processes, since, if this were the case, both the amounts of the acids and the number of counts per 100 gm. of tissue should decrease together and therefore result in no change in the number of counts per millimole. Furthermore, it is very doubtful that this drop is due to lack of absorption of acetate-1-C^{14} into the cell, since the level of radioactivity in the chloroform-mobile (fat, etc.) fractions did not decrease in the diabetic livers. In order for acetate to be converted to the components of this fraction, it obviously must enter the cell.

The results given in Table II thus suggest that some step between acetate and citrate is partially blocked in the liver of the diabetic animal. Since previous work has shown that pyruvate also does not enter the cycle at a normal rate (1), it appears likely that some reaction common to the entry of both pyruvate and acetate is inhibited. The condensation of acetyl CoA with oxalacetate is a reaction common to both. This condensation might be impeded either by a lack of insulin (5) or by a change in the pH of the tissues brought about by diabetic acidosis (6).

The present results appear to be at variance with those of another group of investigators (7) obtained from studies on liver slices *in vitro* from diabetic rats rather than on livers from intact animals as were used in our experiments. These workers reported that more CO_2 was formed, possibly by way of the citric acid cycle, from C^{14}-labeled acetate in diabetic rat liver slices than in slices from normal animals. Insulin decreased acetate oxidation in the diabetic liver slices and proportionally increased the incorporation of acetate into fatty acids. A study of this apparent discrepancy of results is in progress.

Another possible interpretation of the present results is that the rate of phosphate transfer is altered (8), thus affecting the entrance of intermediates into the cycle. The conversion of acetate to acetyl CoA un-

doubtedly would be retarded if the transfer of phosphate were impaired. The formation of thiamine pyrophosphate would also be affected by a slower rate of phosphorylation and this in turn could slow the rate of conversion of pyruvate to acetyl CoA. This makes an interesting hypothesis, since many other reactions alleged to be altered in the diabetic also involve transfer of phosphate groups. Among other steps in metabolism believed to be regulated by insulin are the hexokinase reaction and the transfer of carbohydrate into the cell. Both of these reactions entail phosphorylative changes.

SUMMARY

1. The levels and radioactivities of the acids of the tricarboxylic acid cycle were measured in the livers of normal and alloxan-diabetic rats previously injected with acetate-1-C^{14}.

2. 5 minutes after injection the levels and C^{14} activities of the acids are much lower in diabetic animals than in normal controls.

3. It is postulated that the lower counts in the cycle acids from the livers of diabetic animals result from an inhibition of some reaction necessary for the conversion of acetate to citrate.

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THE RELATION OF BACTERIAL LUCIFERASE TO ALTERNATIVE PATHWAYS OF DIHYDROFLAVIN MONONUCLEOTIDE OXIDATION*

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In publications of Strehler and collaborators (1–3) and McElroy *et al.* (4, 5) some details of the mechanism of luminescence of *Achromobacter fischeri* extracts have been elucidated. Further fractionations of extracts from luminous bacteria have now been made and a complete separation of two enzymes involved in the hydrogen transfers has been accomplished. A study of the inhibition of light production by cytochrome *c* and cytochrome oxidase, and by 2-methyl-1,4-naphthoquinone, has led to a better understanding of the relation of the luminescent system to the respiratory system of the organism. The details of some of these experiments are presented here.

EXPERIMENTAL

The organism *A. fischeri* (ATCC No. 9813) and an unidentified luminescent coccobacillus (No. 9 of Cormier and Strehler (6)), which appears to have a very similar luciferase, were grown and harvested as previously described (7). The acetone-dried powders and extracts were prepared in the same manner as those used by Strehler and Cormier (7).

Cytochrome *c*, reduced diphosphopyridine nucleotide ($DPNH_2$), and flavin mononucleotide (FMN) were obtained from the Sigma Chemical Company, the dodecyl aldehyde from The Matheson Company, Inc., the 2,3-dimercaptopropanol (BAL) from Hynson, Westcott, and Dunning, Inc., and the menadione (2-methyl-1,4-naphthoquinone) was the Eastman Kodak Company's purest grade.

Light measurements were made with the quantum counting device described by Strehler (8), except that a 1P22 photomultiplier was used. For the production of light of a suitable intensity for measurements, the following components were added to make a total volume of 3 ml.: 0.4 mg. of $DPNH_2$, 0.005 mg. of FMN, 0.1 mg. of dodecyl aldehyde, 2.5 ml. of 0.1 M phosphate buffer, pH 7.0, and 0.1 ml. of 2 per cent, by weight, water extract of acetone-dried bacteria clarified by centrifugation for 75 minutes at 39,000 r.p.m. Such a mixture gave approximately 2000 c.p.s. with

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the light-measuring device operated as a pulse counter. The counting rate declined very slowly, a nearly steady rate being maintained for several minutes. Purified luciferase coupled with $DPNH_2$ oxidase can be substituted for the acetone-dried bacterial extract.

Optical density measurements were made with a Beckman spectrophotometer and fluorescence measurements with a Farrand fluorometer.

Protein determinations were done by the method of Lowry *et al.* (9). Samples containing Fe^{59} were measured by direct plating on glass plates and counting was done by the use of an end window Geiger-Müller tube attached to a scaling device.

Fractionation of the extracts was accomplished by means of classical procedures in which $(NH_4)_2SO_4$ was used and by electrophoresis at pH 6.5 on starch columns as described by Kunkel (10).

The *Escherichia coli* extract used as a source of $DPNH_2$ oxidase was the Fraction C of Billen and Volkin (11) prepared by grinding *E. coli* with alumina and extracting with 0.05 M NaCl solution. The solution was clarified by centrifugation at 105,000 $\times$ *g* for 90 minutes.

RESULTS AND DISCUSSION

Separation of the $DPNH_2$-oxidizing system from the luciferase proper was accomplished by overnight dialysis at 5° against 0.02 M phosphate buffer, pH 6.5, of the fraction of bacterial extract soluble in 30 per cent saturated $(NH_4)_2SO_4$ and insoluble in 60 per cent saturated $(NH_4)_2SO_4$ solution. The labile $DPNH_2$ oxidase was destroyed by this procedure. Since the luciferase does not react with $DPNH_2$, it was necessary to conduct assays by addition of a crude $DPNH_2$ oxidase prepared from *E. coli* as described. The luciferase may also be measured, in the absence of $DPNH_2$ oxidase, by addition of menadione together with an excess of BAL to maintain the quinone in the reduced state. The reduced menadione is capable of reducing FMN to provide the $FMNH_2$ necessary for light emission. However, this reversal of menadione reduction is slow and the steady state light production is of low intensity compared with that obtainable with the complete system. Experiments establishing this relation are illustrated in Fig. 1.

Alternatively, the luciferase could be demonstrated by reduction either with $NaBH_4$ or Fe^{++} or with combinations of 2,3-dimercaptopropanol with Fe^{++} or Cu^{++}. The non-enzymic methods are not satisfactory for assay purposes because they produce a light emission of short duration. When combinations of BAL with Fe^{++} or Cu^{++} are used, the duration of emission is governed by the rate of autoxidation of the combination which removes the oxygen necessary for the luciferase reaction. A second burst of light is obtained by shaking the solution with air.

For further purification, the luciferase preparation was placed on a starch

column at pH 6.5 (0.02 M phosphate buffer) and a current of 6 ma. per sq. cm. was applied over a period of 8 hours. The temperature of the starch column was not allowed to exceed 5°. The luciferase, which migrated toward the anode, could easily be identified by its flavin-like fluorescence.

It is now known that several flavoproteins owe part of their catalytic activity to associated elements such as iron or molybdenum. The subject has been reviewed recently by Mahler and Green (12). Although it is

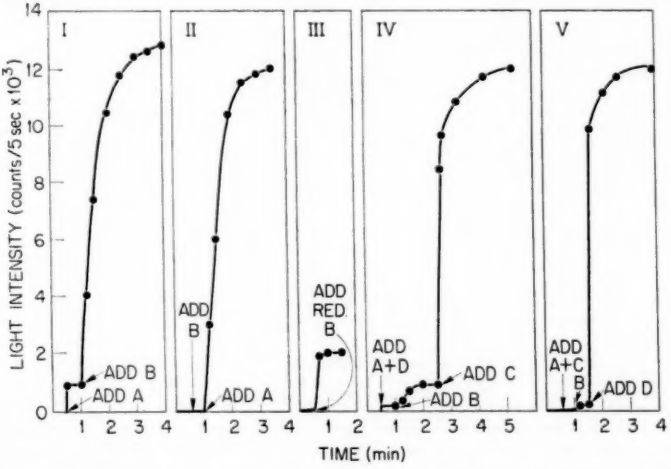

FIG. 1. Effect of reduced menadione on light production in the absence of DPNH and the dependence of this reaction on FMN and long chain aldehydes. The complete system consisted of the following components: 0.4 ml. of a 2 per cent (by weight) water extract of acetone-dried powder of *A. fischeri*, 20 γ of menadione (*B*), 0.003 M (final concentration) BAL (*A*), 5 γ of FMN (*C*), 100 γ of dodecyl aldehyde (*D*), and 2.0 ml. of 0.1 M NaH_2PO_4 (pH 7.0). Final volume, 3 ml. Saturating amounts of *C* and *D* were used in Experiments I, II, and III. *C* and *D* were added to Experiments IV and V as indicated.

improbable that a metal is required by luciferase for its production of light, attempts to demonstrate the presence or absence of these elements seemed advisable. Because of the small amount of luciferase available, it was not possible to obtain unequivocal results with molybdenum. It seemed likely that isotopic iron would provide useful data. Some batches of the coccobacillus were therefore grown on a medium in which the iron was replaced by carrier-free Fe^{59} and the luciferase was subjected to starch column electrophoresis. The results of one of these experiments are shown in Fig. 2, where no evidence may be seen of a radioactive peak in the position occupied by the luciferase.

The fluorescent pigment associated with the luciferase activity was sep-

arated from the active protein by boiling the water solution and subjecting the resulting extract to analysis by paper chromatography with 0.02 M phosphate buffer at pH 8. The R_F was found to be 0.58, in exact agreement with that of an authentic specimen of FMN. Under the same conditions, riboflavin showed an R_F of 0.28. Furthermore, the fluorescence spectra of FMN and the unknown were similar, both having a peak emission at the same wave-length (550 mμ, uncorrected for 1P22 phototube sensitivity). There is, therefore, no reason to doubt that the

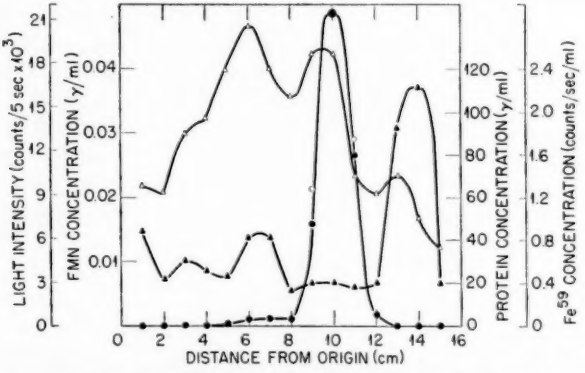

FIG. 2. A comparison of luciferase activity, Fe^{59} content, FMN concentration, and protein concentration from starch electrophoresis column fractions. Luciferase activity determinations were done as described in the experimental procedures. Fraction C (0.05 ml.) of Billen and Volkin (11) was used as the source of $DPNH_2$ oxidase. 1 sq. cm. cuts were extracted from the starch column as indicated and eluted with 2.0 ml. of water. 0.2 ml. of this eluate was used for testing. The luciferase activity was normalized with the FMN in the 10 cm. fraction. ●, luciferase activity; ○, FMN concentration; ▲, Fe^{59} concentration; △, protein concentration.

fluorescent material from the most active fractions is in fact riboflavin monophosphate. The values for FMN, determined by fluorescence measurements, are presented in Fig. 2. Fractions adjacent to those indicated showed insufficient fluorescence for quantitative estimation. The close association of this tightly bound FMN with the luciferase from both organisms in numerous experiments and the correlation of FMN content with luciferase activity make it appear probable that luciferase contains a relatively non-dissociable FMN. Based on the assumption that luciferase contains 1 mole of FMN per 80,000 gm., the most active preparation was at least 10 to 20 per cent pure.

If an estimate of iron content was based on the FMN concentration observed in the most highly purified luciferase fraction and if 1 mole of Fe

were assumed per mole of FMN, radioactivity should have been easily detectable. Since no peak of radioactivity coincided with the peak of luciferase activity, it seems very unlikely that iron is a component of this enzyme.

The effects of cytochrome additions to the unfractionated luminescent system from *A. fischeri* are shown in Fig. 3. A drop in light followed the addition of oxidized cytochrome *c*, but no immediate effect was observed with a cytochrome *c* preparation which had been fully reduced catalytically

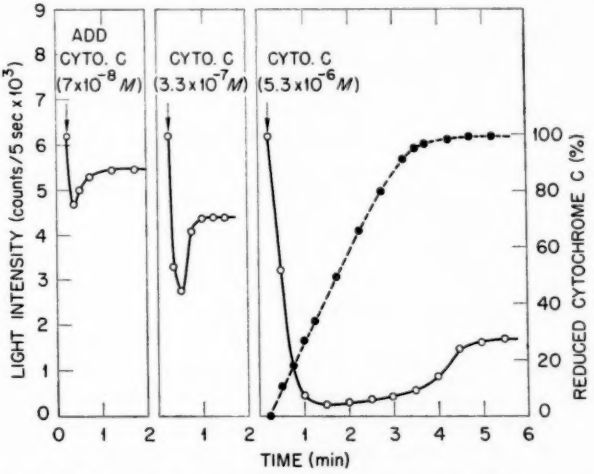

FIG. 3. Effect of oxidized cytochrome *c* on luminescence. The components of the light reaction were the same as those described in the experimental part of the text. Cytochrome *c* was added as indicated. ○, light production; ●, cytochrome *c* reduction.

with H_2 (see Table I). Fig. 3 also demonstrates the changes in absorption at 550 mμ plotted against the same time scale as the light emission. Even with reduction of the cytochrome *c* to the degree that no spectroscopically detectable amount of the oxidized form was present, there was still a significant reduction in light emission. That this reduced light output was caused by a small steady state level of oxidized cytochrome *c* is evident from the results of additional experiments to be described.

The addition of cyanide in the presence of cytochrome *c* increased the light intensity somewhat, whereas exposure to 95 per cent CO-5 per cent O_2 restored it completely. On the other hand, addition of small amounts of mitochondrial preparations containing cytochrome oxidase reduced the light output still further; this reduction was also partially reversed by cy-

anide or BAL alone and almost completely reversed when both substances were added. These data are summarized in Table I. It was not possible to demonstrate conclusively that the action of CO was fully reversed by

TABLE I

Effect of Steady State Level of Oxidized Cytochrome c on Luminescence of Cell-Free Extracts of A. fischeri

Final concentrations of the following materials were added to the steady state luminescent system: KCN (0.0005 M), BAL (0.003 M), rabbit liver mitochondrial preparation (0.1 ml. per 3 ml.), cytochrome *c* (2.7×10^{-6} M), antimycin A (0.2 ml. per 3 ml. of saturated water solution), 95 per cent CO-5 per cent O_2 replacing air. These components were added as indicated.

Experiment No.*	Components added	Light intensity at steady state, per cent of control
I	Control (no addition)	100
	" + BAL	110
	" + KCN	80
	" + mitochondria	98
	" + oxidized cytochrome *c*	59
	" + " " " + KCN	70
	" + KCN + BAL	95
	" + antimycin A	92
	" + reduced cytochrome *c*	94
	" + oxidized " " + BAL	100
	" + mitochrondria + oxidized cytochrome *c*	15
	" + " + " " " + BAL	33
	" + " + " " " + KCN	47
	" + " + " " " + " + BAL	85
	Control + oxidized cytochrome *c* + antimycin A	51
	" + " " " + " " " + mitochondria	15
II	Control	100
	" after evacuation	110
	" " " + 95% CO-5% O_2	100
	" " " + cytochrome *c*	30
	" " " + " " + 95% CO-5% O_2	100

* Experiments I and II were performed with extracts from two different batches of organisms.

light because of the nature of the measurements necessary to do so. It therefore remains inconclusive that the steady state inhibition of light output when cytochrome *c* is added to the bacterial extracts results from a typical cytochrome oxidase, but there is no doubt that the inhibition is reversed by a cyanide- and CO-sensitive mechanism.

The "inhibition" of light output resulting from addition of cytochrome *c* was slowly reversed by BAL. It seemed possible that this effect might be exerted through its action on Slater's factor (13). However, BAL added with mitochondrial preparations did not fully restore light output at concentrations which completely abolished succinoxidase activity. Furthermore, the succinic oxidase inhibitor antimycin A[1] had no effect whatever on light emission either in the presence or absence of added cytochrome *c* and cytochrome oxidase. The reversal of the cytochrome effect is, therefore, probably brought about by reduction of the cytochrome *c* by BAL, a reaction which is too slow to compete successfully with cytochrome oxidase at elevated concentrations.

Since it was shown that the reduction of cytochrome *c* by $DPNH_2$ in the presence of the unfractionated extracts was FMN-dependent, there must be present a "$DPNH_2$-cytochrome reductase" which requires FMN. It is not yet established whether the reaction between $FMNH_2$ and Fe^{+++}-cytochrome *c* in these extracts is enzymic. However, it has been impossible to demonstrate such a reaction at the concentrations used in our experiments in the absence of the extracts. Moreover, if the extracts contain a quinone, the reduction of cytochrome *c* may take place by reaction with the hydroquinone through the "quinone reductase" activity known to be present (14, 15).

Further evidence concerning the nature of the steps leading to light emission was obtained by a study of the dependence of the light reaction on the concentration of FMN and the FMN-dependent oxidation of $DPNH_2$. Data from typical experiments with the crude extract are shown in Fig. 4. It may be seen that the oxidation of $DPNH_2$ follows the usual kinetics up to at least 0.2 mmole of FMN per liter.

On the other hand, light emission reaches an optimal intensity with increasing concentrations of FMN but is inhibited by further additions of FMN (purified by paper chromatography) beyond about 1.5 μmoles per liter. The inhibition by excess FMN was only slightly altered by addition of *E. coli* $DPNH_2$ oxidase. Apparently, FMN competes with $FMNH_2$ for a site on the luciferase protein. Because the purified enzyme appears to contain tightly bound FMN (see Fig. 2), the FMN which must be added to elicit light production probably acts as a hydrogen carrier between $DPNH_2$ and the bound FMN.

The operation of FMN as a hydrogen transport agent between $DPNH_2$ and a flavoprotein is somewhat novel, but there appears to be no theoretical reason why this could not occur. The tightly bound flavin, as indicated by its fluorescence, is normally in the oxidized state and an additional site can be occupied by either FMN or $FMNH_2$ with a many fold lower

[1] We are indebted to Dr. F. M. Strong, Department of Biochemistry, University of Wisconsin, for generously supplying the antimycin A.

dissociation constant for the latter. The FMN-$FMNH_2$-enzyme combination could perhaps contain the 2 moles of flavin in spatial orientation such that the formation of a quinhydrone-like intermediate would be favored. It is tempting to speculate that the long chain aldehyde which is required for light production, or a derivative of it, may help to bind the two flavins and facilitate transfer of the hydrogens or electrons leading to the light emission. The oxidation accompanying light emission returns the $FMNH_2$ to the oxidized state which then dissociates from the enzyme surface. It

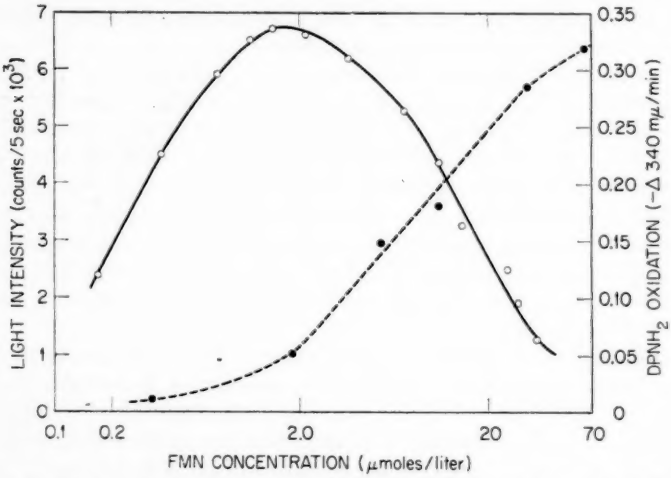

FIG. 4. Effect of FMN concentration on light production and the rate of $DPNH_2$ oxidation. The components of the light reaction were the same as those described in the experimental procedure except that FMN was added as indicated. The complete system for determining the $DPNH_2$ oxidation rate was the same as that for the light reaction. ○, light production; ●, $DPNH_2$ oxidation.

should be pointed out that the two flavins and associated aldehyde can be arranged in such a manner that the resulting complex would resemble structurally the well known chemiluminescent substance, dimethyl diacridylium nitrate. Further work will be required to determine whether such an arrangement does exist.

On the basis of the experimental results the series of reactions involved in these studies are probably best formulated as follows:

(1) $$DPNH_2 + FMN \underset{\text{"}DPNH_2\text{ oxidase"}}{\longleftrightarrow} DPN + FMNH_2$$

(2) $$FMNH_2 + \text{luciferase-FMN} \rightleftharpoons \text{luciferase-FMN-}FMNH_2$$

(3) Luciferase-FMN-$FMNH_2$ $\xrightarrow[RCHO]{O_2}$ luciferase-FMN + FMN + light + ...

The following alternative reactions compete with Reaction 2.

(4) $FMNH_2$ + "quinone" $\rightleftarrows$ FMN + "hydroquinone"

(5) "Hydroquinone" + $2Fe^{+++}$-cytochrome $c \rightarrow$ "quinone" + $2Fe^{++}$-cytochrome $c + 2H^+$

(6) "Hydroquinone" + $O_2 \rightarrow$ "quinone" + H_2O_2

(7) $FMNH_2 + 2Fe^{+++}$-cytochrome $c \rightarrow$ FMN + $2Fe^{++}$-cytochrome $c + 2H^+$

(8) $FMNH_2 + O_2 \rightarrow FMN + H_2O_2$

Reactions 4, 5, 6, 7, and 8 may compete with luciferase for $FMNH_2$. Reactions 4 and 5 have been investigated by Cormier and Totter (14), the quinone used being 2-methyl-1,4-naphthoquinone (menadione). This reaction has now been shown to be reversible, since menadione reduced either catalytically or by BAL leads to a small, steady light production when mixed with the unfractionated luminescent system in the absence of $DPNH_2$. In the oxidized state, the menadione abolishes light output until it is almost completely reduced. The level of light output does not return to the initial value but to a lower steady state value because of the autoxidation reaction (6) of the reduced menadione (Figs. 1 and 5). Reaction 7 has been discussed in preceding paragraphs.

In the absence of excessive competition from cytochrome *c* and quinones and in the presence of excess FMN, luciferase, O_2, and aldehyde, the light output is governed by the rate of transfer of hydrogen from $DPNH_2$ (Reaction 1) and the competing autoxidation of $FMNH_2$. The light output can be increased about 20-fold in the unfractionated extracts by coupling the luciferase with the very active FMN-$DPNH_2$ oxidase from *E. coli*.

The actions of quinones, cyanide, azide, and carbon monoxide on living luminescent bacteria have been investigated by several groups of workers. Harvey (16) has reviewed the subject extensively. The experiments on extracts provide confirmation for the interpretation given by Spruit and Schuiling (17) of the results of their studies of quinone inhibition of bioluminescence in intact bacteria. Our results are also in accord with those of Van Schouwenburg and Van der Burg (18) and Harvey (16) in their researches on the mechanisms of carbon monoxide and cyanide inhibitions, respectively.

Another competing enzyme which is sometimes troublesome when the luminescent system is being used for assay purposes is alcohol dehydrogenase, which catalyzes the reduction of the long chain aldehyde necessary

for light emission. Very small quantities of rabbit liver homogenate contain a sufficient amount of the enzyme to bring about abolition of the light emission in a few seconds. The light is restored momentarily upon a second addition of aldehyde. This reaction provides a very sensitive and rapid procedure for the determination of alcohol dehydrogenase activity.

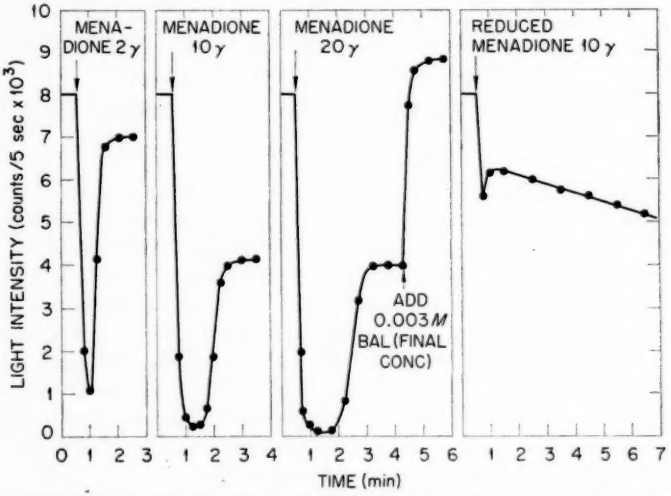

FIG. 5. Effect of oxidized and reduced menadione on light production in extracts of *A. fischeri*. The experimental procedure in the text gives the various components of the light reaction. Menadione, BAL, and reduced menadione were added as indicated.

SUMMARY

A separation of bacterial luciferase from the $DPNH_2$ oxidase required for bacterial extract luminescence was accomplished by destruction of the latter enzyme. A $DPNH_2$ oxidase prepared from *Escherichia coli* was found to substitute for the destroyed enzyme.

Luciferase partially purified by starch column electrophoresis contained tightly bound FMN but appeared not to contain iron, as indicated by tracer experiments.

The luciferase reaction was inhibited by excess FMN. This result was interpreted as suggestive that the light-emitting complex, in part, consists of luciferase apoenzyme with 2 moles of flavin phosphate, only 1 of which is in the reduced state.

Inhibition of luminescence by menadione and by various combinations of cytochrome *c* and preparations containing the cytochrome oxidase sys-

tem were studied. It was concluded that these "inhibitors" act by competing with luciferase for the hydrogen of $FMNH_2$.

Alcohol dehydrogenase reduced light output by rapid reduction of the long chain aldehyde necessary for light production.

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THE INCORPORATION OF CARBOXYL AND BICARBONATE CARBON INTO GLUTAMIC ACID BY THE RAT*

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The isotope pattern found in rat liver glycogen following administration of a variety of carbon-labeled compounds has been used by Wood and his coworkers (1–6) in studies concerning carbon metabolism. Experiments of a similar nature involving glutamic acid as the target molecule will be described in this paper.

Its relative abundance, ease of isolation, and interconversion *in vivo* with α-ketoglutarate, a key intermediate in the Krebs tricarboxylic acid cycle, make glutamate a useful compound for studying metabolic pathways. Such studies with microorganisms (7–11) and rats (12–14) have been described. However, no data are available concerning the complete isotope pattern found in glutamate derived from mammalian tissue following the administration *in vivo* of carbon-labeled substrates.

With the exception of purine synthesis, those *in vivo* mammalian CO_2 fixation reactions which result in synthesis of carbon to carbon bonds involve compounds of the Krebs tricarboxylic acid cycle. The principal reaction is the formation of oxalacetic (15) or malic acid (16). Fixation of $C^{14}O_2$ in this manner gives rise to α-ketoglutarate, and hence glutamate, labeled *only* in carbon 1 (17–21).[1] It would be predicted, therefore, that carboxyl-labeled 4-carbon dicarboxylic acids (oxalacetic), 3-carbon glycolytic acids (pyruvic), or their biological precursors (aspartic acid, alanine) would also give rise to glutamic acid labeled *only* in carbon 1. However, administration[2] of DL-glyceric acid-1-C^{14}, bicarbonate-C^{14} (12), and $C^{14}O_2$ to rats (13), and DL-aspartic acid-4-C^{14} to rabbits,[3] resulted in tissue glutamic acid having as low as 70 to 83 per cent of its total radioactivity located in carbon 1. Since in none of these investigations was a complete degradation of glutamate accomplished, the location of activity other than in carbon 1 is not known.

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[1] The International unit carbon-numbering system for glutaric acid derivatives will be used throughout the presentation. Thus the α-carboxyl of glutamate is designated carbon 1 and γ-carboxyl, carbon 5.

[2] Sallach, H. J., and Rose, W. C., personal communication.

[3] Koeppe, R. E., and Rose, W. C., unpublished data.

To elucidate further the pathway of incorporation of carbon dioxide and carboxyl carbon into glutamate, bicarbonate-C^{14}, DL-alanine-1-C^{14}, and glycine-1-C^{14} have been injected into rats, tissue glutamate has been isolated and degraded, and each carbon assayed for radioactivity. Although most of the radioactivity of the isolated glutamic acid was located in carbon 1, significant activity was also present in carbon 5, while trace amounts were found in positions 2, 3, and 4.

In contrast, administration of acetate-1-C^{14} gave rise to glutamic acid labeled predominantly in carbon 5 and having the remaining one-third of its activity in carbon 1.

EXPERIMENTAL

Male albino rats (of Wistar origin) from our own colony were used in these investigations. The various isotopic compounds were dissolved in saline and administered by intraperitoneal injection. During the period between injection and sacrifice the animals were placed in an all-glass metabolism chamber which was swept with a slow stream of air. The expired CO_2 was trapped in alkali.

Rats 1 through 9 were fed Purina checkers *ad libitum*. Rats 11 and 13 were fed, for 2 weeks prior to injection, a mixture of ground Purina checkers and 10 per cent L-leucine, also *ad libitum*. Their respective litter mates, Rats 10 and 12, were fed ground checkers for the same period. The leucine-fed animals gained weight at a rate very nearly comparable to that of the controls. Rat 9 was partially hepatectomized 3 days before injection.

The isotopic compounds used were DL-alanine-1-C^{14} (Tracerlab, Inc., 1.06 mc. per mmole), sodium acetate-1-C^{14} (Isotopes Specialties Company, Inc., 2.0 mc. per mmole), glycine-1-C^{14} (Isotopes Specialties, 0.82 mc. per mmole), and sodium bicarbonate-C^{14} (Nuclear Instrument and Chemical Corporation, 1.54 mc. per mmole).

Data concerning the quantities injected and the duration of experiments are given in Tables I and II.

Isolation Procedures—Immediately after sacrifice of the rat by a blow on the head, the liver was removed and a protein powder prepared from the liver and "carcass" by conventional means involving extraction of the ground tissue with 10 per cent trichloroacetic acid, acetone, and ether. "Carcass" refers to the entire animal, except liver, including the washed gastrointestinal tract.

The carcass protein was hydrolyzed by boiling under a reflux for 24 hours with 10 volumes of 6 N HCl. After filtration with Celite and removal of excess HCl by vacuum distillation, the hydrolysate was brought to pH 2.2 by addition of the free base form of a weak anion resin (Amberlite IR-45,

Rohm and Haas) and treated with charcoal (Nuchar) (22). Glutamic and aspartic acids were then quantitatively removed from the charcoal filtrate by passage through an Amberlite IR-45 column in the acetate cycle.

A partial separation of aspartic and glutamic acids was achieved when the amino acids were eluted from the column with 1 N acetic acid. After evaporation to dryness of the eluates containing glutamic acid, the residue was dissolved in a small volume of water and glutamic acid hydrochloride crystallized by saturation with HCl; crystalline glutamic acid was obtained by neutralization with pyridine and precipitation with ethanol.

Sufficient L-glutamic acid was added to the liver protein powders of Rats 1 to 9 to dilute 10-fold the 12.2 per cent glutamate reported present in rat liver protein (23). This mixture was then hydrolyzed by boiling under a reflux with 6 N HCl. The hydrolysate was concentrated *in vacuo* to a small volume, decolorized with charcoal, and glutamic acid hydrochloride crystallized by saturation with HCl. Free glutamic acid was then obtained as described above.

Glutamic acid was isolated, without addition of carrier, from the liver protein of Rats 10 to 13 by a method identical to that described above for carcass glutamate. Carrier L-glutamic acid was then added prior to recrystallization and degradation.

Each glutamic acid sample was characterized by paper chromatography, C analysis, and ninhydrin-released CO_2. With the exception of the liver glutamates of Rats 1, 3, and 4, all samples were recrystallized to constant specific activity.

Degradation Procedures—Carbon 1 of glutamate was liberated as CO_2 with ninhydrin and measured manometrically as described by Van Slyke *et al.* (24).

Glutamic acid was completely degraded by the method of Mosbach, Phares, and Carson (25, 26). This procedure selectively converts each carbon of glutamate to CO_2. The authors have experienced only minor difficulties in repeating this excellent method of degrading glutamate. Yields of butyric acid have been found to be more consistent if a pH of 6.5 to 7.0 is reached prior to treatment with chloramine-T. Also, it has been helpful, following oxidation of amines, to remove excess permanganate by the addition of $FeSO_4$. As a further check on radioactivity in carbon 5, we have run Schmidt reactions (25) on glutamic acid by using a 3-fold excess of azide. Approximately 50 to 70 per cent yields of CO_2 from carbon 5 were obtained by this procedure. Recently Miller and Bale (27) have described a similar reaction.

Combustion Methods—The glutamate samples isolated from Rat 1 were converted to CO_2 by the persulfate wet oxidation technique of Anthony and Long (28). All others were oxidized by the macrocombustion method of

Van Slyke *et al.* (29, 30). The amount of CO_2 liberated during degradation and combustion of tissue glutamates isolated from Rat 1 was determined as $BaCO_3$ (28), whereas that obtained from all subsequent degradations and combustions was measured manometrically according to the method of Van Slyke and Folch (29).

Assay of Radioactivity—All measurements of radioactivity were made with a vibrating reed electrometer (Applied Physics Corporation, Pasadena, California). The $BaCO_3$ samples obtained from Rat 1 were treated with H_3PO_4 and the liberated CO_2 introduced into an ion chamber as described by Anthony and Long (28). The CO_2 measured manometrically was transferred directly from the Van Slyke apparatus to the ion chamber via a Drierite trap and mercury valve (28, 31). Reproducible data have been obtained readily in this laboratory by use of the wet oxidation manometric technique for total C and the vibrating reed electrometer for radioactivity assay. Liquid nitrogen and dry methane, two essentials of the Van Slyke, Steele, and Plazin method (32), are not required in this procedure, yet the times of analysis and assay are essentially the same. It should be emphasized, however, that samples analyzed in our laboratory have always contained more than 2 mg. of C. Whether equally precise results would be obtained with smaller samples has not been determined.

RESULTS AND DISCUSSION

Results of the glutamate degradations are presented in Tables I and II. The data from Rat 10 differ strikingly from those of the other bicarbonate-treated animals and will be discussed separately. No significant differences were found in the labeling patterns of corresponding liver and carcass glutamates. The data for carcass glutamic acids, which are more complete and more consistent from one animal to another, have been used as the basis for most of the following discussion.

With the exception of those rats which received acetate-1-C^{14}, most of the C^{14} in the rat glutamates was located, as expected, in carbon 1. The high percentage of labeling in carbon 1 is in general agreement with results cited earlier[2, 3] (12, 13). However, *all* these samples also had appreciable radioactivity in carbon 5. Although in most instances definite activity was detected in positions 2, 3, and 4, the amounts were often small in comparison to the background and hence subject to considerable error. Indeed, the activity found in these carbons is reminiscent of that found in carbons 1, 2, 5, and 6 of liver glycogen by Shreeve *et al.* (33) after administration of bicarbonate-C^{14}. How carboxyl or bicarbonate carbon is incorporated into these positions of glutamate and glycogen is not clear. It should be emphasized, however, that the small amount of activity found in position 2, as compared to 5, precludes any considerable conversion *in vivo* of succinate to α-ketoglutarate.

The finding of significant amounts of radioactivity in carbon 5 following administration of bicarbonate-C^{14} is, to our knowledge, the first demonstration of a CO_2 fixation *in vivo* into this carbon of glutamate. Any mechanism whereby CO_2 is fixed into the carboxyl of acetate would explain labeling in carbon 5 (2, 34). Bicarbonate might be incorporated into the carboxyl

TABLE I

C^{14} Distribution in Rat Glutamic Acid after Administration of $NaHC^{14}O_3$

		Rat 9*		Rat 10		Rat 11†		Rat 12		Rat 13†	
Rat weight, *gm.*		130		155		152		185		170	
Dose injected, *μc.*		100		150		150		150		150	
Duration, *hrs.*		6.5		4.0		4.0		4.0		4.0	
Per cent injected, exhaled as CO_2		85		76		87		85		92	
		C^{14} in glutamic acid									
		mμc. per mM	*per cent total*	*mμc. per mM*	*per cent total*	*mμc. per mM*	*per cent total*	*mμc. per mM*	*per cent total*	*mμc. per mM*	*per cent total*
Carcass	Total	13.0		37.5		17.8		14.0		18.2	
	Carbon 1, COOH	11.4	88	19.8	53	15.1	85	11.3	81	15.1	83
	" 2, $CHNH_2$			2.7	7.2			0.22	1.6	0.25	1.4
	" 3, CH_2			2.7	7.2			0.22	1.6	0.29	1.7
	" 4, CH_2			4.1	10.9			0.35	2.5	0.55	3.0
	" 5, COOH	1.22	9.4	7.8	20.8	1.93	10.8	1.52	10.9	1.89	10.4
	Sum,‡ carbons 1–5			37.1	99			13.6	97	18.1	99
Liver§	Total	69		138		78		54		56	
	Carbon 1, COOH	60	87	91	65	70	90	46	85	50	85
	" 5 "	3.5	5.1	22.4	16.2			4.4	8.2	3.3	5.9

* Partially hepatectomized 3 days before the experiment.

† For 2 weeks before the experiment these rats were fed a diet supplemented with L-leucine to the extent of 10 per cent.

‡ The values in the per cent total columns were obtained by dividing the sum of the millimicrocuries per millimole found in each carbon by the total millimicrocuries per millimole and multiplying by 100.

§ The values from Rats 9, 12, and 13 are corrected for a 10-fold dilution with carrier; those from Rats 10 and 11 for a 20-fold dilution.

of acetate via the conversion of succinate to "acetyl" (35, 36) or the fixation of CO_2 into the carboxyl of acetate during the metabolism of leucine and isovalerate (37–39). Rats 11 and 13 were fed a high leucine diet prior to injection of bicarbonate in an effort to increase CO_2 fixation into carbon 5. Although the expected increase was not observed, the results should not be construed as evidence against the Coon mechanism of leucine metabolism. Much of the ingested leucine may have been excreted as a conjugate or may have been so rapidly oxidized that at the time of injection leucine metabolism had returned to normal. Recently, Hendler and Anfinsen (40)

TABLE II

C^{14} Distribution in Rat Glutamic Acid after Administration of Alanine-1-C^{14}, Glycine-1-C^{14}, and Acetate-1-C^{14}

		Alanine-1-C^{14}						Glycine-1-C^{14}				Acetate-1-C^{14}			
		Rat 1		Rat 3		Rat 4		Rat 6		Rat 8		Rat 5		Rat 7	
Rat weight, *gm.*		222		185		99		120		127		130		127	
Dose injected, *μc.*		101		129*		103		105		115		123		120	
Duration, *hrs.*		6.3		24.0		6.0		6.0		6.0		6.0		6.0	
Per cent injected, exhaled as CO_2		66		79		72		29		36		64		78	
		C^{14} in glutamic acid													
		mμc. per mM	*per cent total*	*mμc. per mM*	*per cent total*	*mμc. per mM*	*per cent total*	*mμc. per mM*	*per cent total*	*mμc. per mM*	*per cent total*	*mμc. per mM*	*per cent total*	*mμc. per mM*	*per cent total*
Carcass	Total	9.4		15.9		20.7		8.3		10.3		172		270	
	Carbon 1, COOH	8.8	94	14.2	89	18.6	90	6.9	83	8.3	81	53	31	83	31
	" 2, $CHNH_2$	0.1	1	0.29	1.8	0.19	0.9	0.06	0.7	0.04	0.4			0.28	0.10
	" 3, CH_2	0.1	1	0.26	1.6	0.19	0.9	0.05	0.6	0.08	0.8			0.38	0.14
	" 4, "	0.1	1	0.17	1.1	0.32	1.5	0.12	1.4	0.12	1.2			1.55	0.57
	" 5, COOH	0.32	3.4	0.51	3.2	1.62	7.8	0.99	11.9	1.39	13.5	112	65	178	66
	Sum,‡ carbons 1–5	9.4	100	15.4	97	20.9	101	8.1	98	9.9	96			263	97
Liver†	Total	77		101		78		58		66		153		209	
	Carbon 1, COOH	74	96	91	90	66	85	51	88	62	94	54	35	72	34
	" 5, "	1.2	1.6	1.3	1.3	3.8	4.9					96	63	129	62

* Administered in three equal portions, 3 hours apart.

† These values are corrected for a 10-fold dilution with carrier.

‡ See foot-note to Table I.

have reported the incorporation of $C^{14}O_2$ into carbon 5 of glutamate by minces of hen oviduct.

Partial hepatectomy prior to bicarbonate administration (Rat 9) caused no striking change in labeling of either carcass or liver glutamic acid.

It is of interest to compare the labeling patterns produced by the carboxyls of alanine and glycine with those of bicarbonate. The carboxyl carbons may be converted to glutamate via the bicarbonate pool or by a more direct pathway. Prior conversion to bicarbonate would result in a specific activity in glutamate *less* than that observed with bicarbonate-C^{14}.

TABLE III

Corrected Specific Activity of Carcass Glutamic Acid Carbons after Administration of $NaHC^{14}O_3$, Alanine-1-C^{14}, and Glycine-1-C^{14}

Rat No.	Compound administered	Correction factor*	Corrected specific activity, mμc. per mmole					
			Total C^1	Carbon 1, COOH	Carbon 2, $CHNH_2$	Carbon 3, CH_2	Carbon 4, CH_2	Carbon 5, COOH
9†	$NaHC^{14}O_3$	1.53	19.9	17.5				1.87
10	"	1.36	51.0	27.0	3.7	3.7	5.6	10.6
11‡	"	1.17	20.8	17.7				2.26
12	"	1.44	20.2	16.3	0.32	0.32	0.50	2.19
13‡	"	1.23	22.4	18.6	0.31	0.36	0.68	2.33
1	Alanine-1-C^{14}	3.33	31.3	29.3	0.3	0.3	0.3	1.1
3	"	1.76	28.0	25.0	0.51	0.46	0.30	0.90
4	"	1.34	27.7	25.0	0.25	0.25	0.43	2.17
6	Glycine-1-C^{14}	3.98	33.1	27.5	0.24	0.20	0.48	3.94
8	"	3.14	32.3	26.1	0.13	0.25	0.38	4.23

* Obtained by dividing the rat weight by the microcuries of $C^{14}O_2$ exhaled.

† This rat was partially hepatectomized 3 days before the experiment.

‡ For 2 weeks before the experiment these rats were fed a diet supplemented with L-leucine to the extent of 10 per cent.

However, incorporation into glutamate without previous formation of bicarbonate might lead to a higher specific activity in glutamic acid. Since the animal weights, amounts of activity administered, and rates of C^{14} excretion are not the same, the data for alanine, glycine, and bicarbonate are more readily analyzed if first corrected for the total bicarbonate-C^{14} available to each gm. of tissue during the course of the experiment. The microcuries of C^{14} excreted as CO_2 per gm. of body weight of animal are roughly proportional to the correction mentioned above. In Table III, the specific activities of the carcass glutamates have been multiplied by the microcuries excreted per gm. of body weight. The "corrected" data are remarkably constant for each group of animals, Rat 10 again being the exception.

If alanine-1-C^{14} and glycine-1-C^{14} are converted to glutamate exclusively through bicarbonate, the "corrected" data should be nearly identical for all animals. However, the data indicate that the carboxyls of alanine and glycine yield higher activity in carbon 1 of glutamate than does bicarbonate. This may be explained by the fact that these amino acids can be converted to carboxyl-labeled pyruvate. The latter can give rise to glutamate labeled in carbon 1 (via oxalacetate and fumarate) without prior conversion to bicarbonate. The carboxyl of glycine appears to be a better precursor of carbon 5 than bicarbonate or alanine. This observation is compatible with a rather direct conversion of small amounts of glycine to acetate and offers a plausible explanation for the conversion of glycine-1-C^{14} to acetate as reported by Sprinson and Coulon (41).

The results with acetate-1-C^{14} are in precise agreement with current concepts of acetate metabolism. A carbon 5 to carbon 1 ratio of 2:1 would be observed after one tour of the Krebs tricarboxylic acid cycle. Wang *et al.* (10) have obtained comparable results with yeast. It is apparent that glutamate, in contrast to glycogen, can be used to distinguish between metabolic pathways proceeding through the carboxyl of the 2-carbon fragment and those proceeding through the carboxyl of the "trioses."

The data from Rat 10 are difficult to explain. Careful analysis of this experiment has led the authors to conclude that these apparently anomalous data did not result from experimental error. Although the specific activity of glutamate from Rat 10 is about double that of other bicarbonate-treated animals, the activity in carbon 1 is only slightly greater. The major difference, a 10-fold one, is in carbons 2, 3, and 4. CO_2 fixation leading to a 2,3-labeled triose would explain the results. Recent reports by Horecker *et al.* (42, 43) provide at least plausible explanations for incorporation of CO_2 into the 2, 3 positions of trioses. However, the magnitude of this incorporation by Rat 10 is surprising and represents the first demonstration of a CO_2 fixation *in vivo* into *all* positions of glutamate.

SUMMARY

The pattern of labeling in rat glutamate has been used to study *in vivo* pathways of carbon metabolism.

Bicarbonate-C^{14} has been found to yield glutamic acid labeled predominantly in carbon 1. Significant amounts of C^{14} were also observed in carbon 5, probably indicating CO_2 fixation into the carboxyl of acetate. Feeding a high leucine diet did not enhance the incorporation of bicarbonate into carbon 5. With the exception of one rat, which incorporated significant amounts of bicarbonate into all carbons of glutamate, only trace amounts of radioactivity were found in positions 2, 3, and 4 of the isolated glutamates.

The labeling patterns observed in tissue glutamic acid following administration of DL-alanine-1-C^{14} and glycine-1-C^{14} were similar but not identical to those observed with bicarbonate.

Acetate-1-C^{14} yielded glutamate with 65 to 70 per cent of its activity in carbon 5, most of the remaining activity being in carbon 1.

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THE METABOLISM OF P^{32}-LABELED RIBONUCLEOTIDES IN TISSUE SLICES AND CELL SUSPENSIONS*

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A number of reports have appeared on studies of the incorporation of ribonucleotides, labeled with C^{14} in the base moiety, into the nucleic acids of yeast (1), bacteria (2, 3), rat liver slices (1), and regenerating liver of rats *in vivo* (4). However, in the instances in which incorporation was actually observed, it has been impossible to ascertain whether the intact nucleotide molecule was built into the nucleic acid structure or whether dephosphorylation and loss of ribose occurred during the process. Recently, Roll *et al.* (5, 6) have studied the incorporation of the phosphorus of P^{32}-labeled purine ribonucleoside-2′- and 3′-phosphates into the ribonucleic acid (RNA) of rat viscera *in vivo;* their data indicate that no significant incorporation of the intact nucleotides occurred.

For some time it has appeared doubtful that phosphorylated compounds, such as hexose and triose phosphates, are able to pass through cell membranes (7–11). It seems more likely that they are dephosphorylated at the membrane surface, after which their component parts may enter separately into the intracellular fluids. As a continuation of investigations in this laboratory concerning the biosynthesis of nucleic acids (12–14), it was of interest to investigate the uptake of nucleotides by certain cells. Accordingly, studies were made of the metabolism of P^{32}-labeled ribonucleoside-2′-, 3′-, and 5′-phosphates in various preparations of normal and neoplastic tissues *in vitro*.

EXPERIMENTAL

Preparation of Labeled Nucleotides

A series of flasks was prepared, each containing five slices (approximately 200 mg.) of 10 day-old ventral subcutaneous transplants of Flexner-Jobling carcinoma from female rats, 200 μc. of radioorthophosphate,[1] and 6 ml. of the medium described by Robinson (15), buffered to pH 7.4. Incubation

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[1] Obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

was carried out in oxygen with gentle agitation for 3 hours at 38°. The entire contents of the flasks were then homogenized, and the nucleic acids were isolated as the sodium nucleates by the procedure previously described (13). These were then hydrolyzed to the 2′- and 3′-nucleotides with alkali (13), or to the 5′-nucleotides with diesterase (16). The hydrolysis products were separated by gradient elution chromatography on Dowex 1 anion exchange resin (formate form) (17).

Adenosine-5′-phosphate of high specific activity was obtained from the acid-soluble fraction of regenerating rat liver. A rat which had been partially hepatectomized 24 hours previously was injected subcutaneously with 8.6 mc. of P^{32}. After 125 minutes the rat was sacrificed and the acid-soluble adenylic acid isolated as described by Hurlbert *et al.* (18); its specific activity was 1.7×10^6 c.p.m. per μmole.

Incubation of Labeled Nucleotides with Tissue Slices and Cell Suspensions—The following tissue preparations were incubated with various samples of labeled nucleotides: (*a*) rat liver slices; (*b*) slices of 10 day-old transplants of rat Flexner-Jobling carcinoma; and (*c*) cell suspensions of 6 day-old transplants of Ehrlich mouse ascites carcinoma (19).

The conditions for the incubations were similar to those described for the preparation of the labeled nucleotides. Slices were incubated for 3 hours and the ascites cell preparations for 2 hours. For purposes of comparison, identical experiments were carried out with inorganic radiophosphate at various levels in place of the nucleotides. At the end of the incubation period the tissue was removed from the medium and washed three times with ice-cold isotonic saline, after which the nucleic acid nucleotides, and in some cases the acid-soluble nucleotides, were isolated as before.

Determination of Specific Activities—Inorganic phosphate and the total phosphorus of the nucleic acids, nucleotides, and acid-soluble fractions were determined by the Fiske-Subbarow method as modified by LePage (20). In the experiment involving adenylic acid of high specific activity, concentrations of nucleotides were determined spectrophotometrically by using empirical constants (*cf.* (18, 21)). P^{32} was counted in solution with dipping counters and corrected for decay and for carrier when appropriate.

Preparation of Phosphodiesterase—Phosphodiesterase was prepared from rattlesnake (*Crotalus adamanteus*) venom by a combination of the methods of Hurst and Butler (22) and of Sinsheimer and Koerner (23). Considerable variation among different batches of venom was observed with respect to the content and ease of separation of phosphomonoesterase and phosphodiesterase; no one method of purification was successful in all cases. The fractions were assayed by the "uranium precipitation" method of Hurst *et al.* (24), a purified sample of commercial RNA being used as substrate. Total and inorganic phosphate of the "uranium-soluble" portion

was determined. The usual assay procedure for monoesterase (22, 23), with adenylic acid as substrate, was found to be misleading when the preparation is to be used for the hydrolysis of RNA, as the oligonucleotides formed during RNA hydrolysis are apparently dephosphorylated by the enzyme more rapidly than are mononucleotides.

RESULTS AND DISCUSSION

The extent of incorporation of the P^{32} from 2′- and 3′-nucleotides and from inorganic phosphate into the nucleic acid and acid-soluble fractions of liver and tumor slices is given in Table I. It may be seen that the intracellular P^{32} was diluted to a lesser degree from inorganic phosphate than from 2′- and 3′-nucleotides. Since a pool of endogenous intracellular 2′- and 3′-nucleotides of a magnitude even remotely approaching that of inorganic phosphate has never been found in any tissue (*cf.* Hurlbert and Potter (25)), it must be inferred that the dilution of the acid-soluble P^{32} was due to the breakdown of the nucleotides to inorganic phosphate, which then became mixed with buffer inorganic phosphate. The fact that the P^{32} from inorganic phosphate and from 2′- and 3′-nucleotides was incorporated into the slices to about the same degree would indicate either that the permeability of the cells to the nucleotide and phosphate was of the same order of magnitude or that extensive dephosphorylation of the nucleotides occurred exterior to the cell membranes. The data for the nucleic acid phosphorus of the slices were a reflection of those for the acid-soluble phosphorus.

The results of similar experiments with suspensions of Ehrlich ascites carcinoma cells (Table I) were somewhat different from those in the tissue slices. Although the extent of incorporation of P^{32} from inorganic phosphate and from 2′- and 3′-nucleotides into the nucleic acids was of the same order of magnitude, the relative specific activities of the nucleic acids derived from inorganic radiophosphate were only slightly higher than those in which the labeled nucleotides were the P^{32} source. In contrast to the tissue slice data, radioactivity was incorporated to a greater extent into the acid-soluble fraction from inorganic phosphate than from the 2′- and 3′-nucleotides. The relative specific activities of the acid-soluble fractions were higher in the cases in which inorganic radiophosphate was incubated than in those involving the labeled nucleotides.

In Table I are also presented the results of experiments in which labeled 5′-nucleotides were employed. It will be seen that the data for this class of compounds differ greatly from those for the 2′- and 3′-nucleotides in the same type of cells. Here both the total incorporation into the nucleic acids and their relative specific activities were greater with the 5′-nucleotides as the precursors than with inorganic phosphate. In the acid-soluble frac-

TABLE I

Distribution of P^{32}

P^{32} source				Nucleic acid P		Acid-soluble P	
Compound*		Radioactivity	Specific activity	Incorporation, as per cent of total	RSA† of P	Incorporation, as per cent of total	RSA† of P
		c.p.m. per mg. tissue	*c.p.m. per γ P*				
Liver slices	A-2′-P	41	230	0.02	0.00	3.2	0.45
	A-3′-P	52	240	0.04	0.01	3.2	0.54
	G-3′-P	99	220	0.01	0.01	3.1	0.84
	C-2′-P	58	220	0.06	0.02	3.7	0.72
	C-3′-P	83	250	0.02	0.01	2.6	0.75
	U-2′-P + U-3′-P	56	270	0.04	0.01	3.2	0.62
	IP	700	290	0.03	0.08	2.1	9.4
	"	70	29	0.06	0.16	2.3	7.2
Flexner-Jobling carcinoma slices	A-2′-P	41	230	0.15	0.02	2.8	0.30
	A-3′-P	52	240	0.11	0.02	3.5	0.47
	G-3′-P	99	220	0.07	0.02	2.7	0.61
	C-2′-P	58	220	0.01	0.03	3.8	0.56
	C-3′-P	83	250	0.07	0.02	2.3	0.45
	U-2′-P + U-3′-P	56	270	0.08	0.02	2.3	0.64
	IP	700	290	0.06	0.14	3.5	16.0
	"	70	29	0.07	0.19	2.0	9.0
Ehrlich ascites carcinoma cell suspensions	A-2′-P + A-3′-P	35	52	0.67	0.18	0.1	0.22
	G-2′-P + G-3′-P	420	230	0.39	0.27	1.6	11.0
	C-2′-P + C-3′-P	66	78	0.25	0.31	0.2	1.20
	U-2′-P + U-3′-P	86	170	0.61	0.12	2.7	4.40
	A-5′-P	80	81	5.6	1.3	2.3	7.00
	C-5′-P	220	130	4.1	2.7	3.0	15.0
	U-5′-P	130	140	0.70	0.30	2.8	7.90
	IP	600	180	0.21	0.39	4.0	13.0
	"	200	60	0.28	0.43	5.2	18.0

* A, adenosine, G, guanosine, C, cytidine, U, uridine, IP, inorganic phosphate.

† RSA, relative specific activity = (specific activity × 100)/(specific activity of precursor).

tion, the uptake of P^{32} from the 5′-nucleotides was less than that from inorganic phosphate. However, the specific activities in the two series were in the same range. It is also evident that the total incorporation of isotope into both the nucleic acids and the acid-insoluble fractions was greater from the 5′- than from the 2′- and 3′-nucleotides. Similarly, the relative specific activities of both fractions were generally higher when derived from 5′-nucleotides than when 2′- and 3′-nucleotides were employed as precursors.

Sufficient radioactivity was obtained in the nucleic acids isolated from the ascites cells after incubation with P^{32}-labeled adenosine-5′-phosphate and cytidine-5′-phosphate to make the results of degradation with phosphodiesterase significant, despite the large losses incurred during this operation. It was found that with either adenylic or cytidylic acid as the P^{32} source, randomization of labeling occurred among the nucleic acid nucleotides. Thus, when adenylic acid had been incubated, the relative specific activities of the RNA nucleotides were adenylic acid 1.6, guanylic acid 0.0, cytidylic acid 1.1, uridylic acid 3.3. With cytidylic acid, the relative specific activities were 2.3, 0.0, 1.2, and 0.0, respectively. This clearly demonstrates that direct incorporation of neither nucleotide had occurred.

Several systems have been described (7–11) in which phosphorylated compounds such as sugar phosphates, adenosine di- and triphosphates, phenylphosphate, and glycerophosphate are unable to penetrate cell membranes, but are attacked by phosphatases which are apparently located on the cell surfaces. As it seemed likely that an analogous situation obtained here, an experiment was designed to demonstrate this. Adenosine-5′-phosphate of high specific activity was incubated as before with ascites cells. The inorganic phosphate and nucleotide components of both the extracellular incubation medium and the acid-soluble fraction of the cells were isolated. As seen in Table II, over 80 per cent of the radioactivity which had been added as adenosine-5′-phosphate remained in the medium at the end of the experiment. The two major radioactive compounds in the extracellular medium were inorganic phosphate and adenylic acid; of the total radioactivity of the extracellular fluid, 73 per cent was isolated chromatographically as inorganic phosphate and less than 0.5 per cent as adenylic acid. The specific activity of the extracellular inorganic phosphate was identical with that calculated by assuming that the P^{32} from 4.2 μmoles of adenylic acid was diluted by the 48.3 μmoles of buffer inorganic phosphate present in the medium. The specific activity of the adenylic acid of the extracellular medium was reduced to 77 per cent of its original value.

The relative specific activities of the intracellular acid-soluble nucleotides

and inorganic phosphate were of the same order of magnitude. That of adenylic acid was slightly higher than that of inorganic phosphate, while the remainder of the acid-soluble nucleotides had lower specific activities. The RNA nucleotides and deoxyribonucleic acid (DNA) were found to be of a very low order of specific activity.

It is evident from these data that most of the adenosine-5′-phosphate added to the medium was dephosphorylated without penetrating the cellular membranes, indicating that phosphatases capable of attacking nucleo-

TABLE II

Distribution of P^{32} from Adenosine-5′-phosphate in Ehrlich Ascites Carcinoma Cell Suspensions

Compound*		RSA*	Fraction of original radioactivity
			per cent
Starting material	A-5′-P, 4.2 μmoles	100	100
	IP, 48.3 μmoles	0	0
Extracellular fraction		6.9	83
	A-5′-P	77	0.34
	IP	8.7	60
Intracellular acid-soluble fraction			10
	A-5′-P	6.3	
	C-5′-P	1.0	
	U-5′-P	4.5	
	IP	5.2	
RNA nucleotides	Adenylic acid	0.02	
	Guanylic "	0.03	
	Cytidylic "	0.03	
	Uridylic "	0.02	
DNA		0.02	

* See Table I, foot-notes.

tides are present on the peripheries of the cells. That such dephosphorylation may be reversible is indicated by the decrease in specific activity of the adenylic acid in the medium; an alternative explanation of this phenomenon would be that a small amount of the cell content might have been emptied into the medium by rupture of some cells.

The fact that the acid-soluble adenylic acid had a relative specific activity slightly in excess of that of the acid-soluble inorganic phosphate may be indicative of a very small penetration of intact adenosine-5′-phosphate into the cell. It is evident, however, that most of the labeled adenylic acid of the intracellular acid-soluble fraction was formed from the intracellular inorganic phosphate. The relatively high specific activities of the

pyrimidine nucleotides of this fraction demonstrate that vigorous phosphorylation was taking place within the cells.

It is clear, because of the randomization of label among the nucleotides, that it would be impossible to demonstrate the incorporation of intact nucleotides into nucleic acids in these cellular systems.

The present data are consistent with the results of the whole animal experiments of Roll *et al.* (5, 6) in which evidence for the incorporation of intact nucleotides into nucleic acids could not be obtained. Their data indicate that the P^{32} from the injected purine ribonucleoside-2′- and 3′-phosphates was randomized among the nucleotides derived from the visceral nucleic acids by alkaline hydrolysis. In the course of the transport of the phosphorylated compounds from the peritoneal cavity to the visceral organs, there are at least two cellular boundaries to be crossed, which, according to the present data, would provide abundant opportunity for the dephosphorylation of the administered nucleotides.

That nucleotides are intermediates in the biosynthesis of nucleic acids remains, however, an attractive hypothesis. Previous work in this laboratory (13) has shown that P^{32} is incorporated into the RNA nucleotides of normal rat liver to a much greater extent than is glycine-2-C^{14}, simultaneously administered, which suggests that the phosphorus is incorporated into a precursor pool at an earlier time than is the glycine. Buchanan and Schulman (26) and Greenberg (27, 28) have demonstrated the importance of nucleotides in the biosynthesis of purines. Potter and associates (25, 29, 30) and Edmonds and LePage (31) have shown the rapid incorporation of various precursors into the acid-soluble nucleoside-5′-phosphates and their derivatives in liver and tumor, and a subsequent decline in specific activities of the acid-soluble nucleotides with simultaneous increase in those of the nucleic acids.

If nucleotides are actually precursors of the nucleic acids, direct proof must most likely await the development of suitable cell-free systems in which adequate nucleic acid biosynthesis can be demonstrated.

The authors wish to express their thanks to Dr. G. A. LePage and to Mr. Robert Keller for assistance with the transplantation of tumors and the preparation of tissues for incubation, and to Mrs. David Gilboe for some of the P^{32} counting.

SUMMARY

P^{32}-labeled ribonucleoside-2′-, 3′-, and 5′-phosphates have been incubated with slices of rat liver and Flexner-Jobling carcinoma, and with suspensions of Ehrlich ascites carcinoma cells. The distribution of the label in the nucleic acid and acid-soluble fractions has been studied. Evi-

dence is presented that nucleotides are dephosphorylated by these systems, and that the observed uptake of P^{32} into the nucleic acids is due to the incorporation of the inorganic phosphate resulting from the nucleotide breakdown.

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A STUDY WITH O^{18} OF ADENOSINE TRIPHOSPHATE FORMATION IN OXIDATIVE PHOSPHORYLATION*

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In a previous communication from this laboratory (1) a reaction of inorganic phosphate with water was described which occurred during phosphorylation coupled to oxidation in the electron transport system in liver mitochondria. This reaction was followed by observing the replacement of O^{18} in labeled inorganic phosphate by normal oxygen from water concomitant with the occurrence of phosphorylation. The results indicated that this inorganic phosphate-oxygen replacement reaction was intimately related to the phosphorylation mechanism since it occurred only when phosphorylation associated with the electron transport system was proceeding and was suppressed by the same means as phosphorylation, for example addition of 2,4-dinitrophenol.

In order to gain further insight into the mechanism of oxidative phosphorylation, the present study was undertaken to follow simultaneously the kinetics of this inorganic phosphate-oxygen replacement reaction and of the transfer of the oxygen-labeled inorganic phosphate to adenosine triphosphate (ATP).[1] The results indicate that, in addition to the inorganic phosphate-oxygen replacement reaction already described, there is a further replacement of oxygen in the sequence of reactions leading to the formation of ATP in oxidative phosphorylation. The same pattern has been observed in our experiments on the inorganic phosphate-ATP exchange reaction described by Boyer and coworkers (2); namely, more oxygen has been replaced in the phosphate transferred to ATP than in the inorganic phosphate itself.

The present study includes a comparison of the kinetics of the inorganic phosphate-oxygen replacement reaction and the transfer reaction to ATP with (*a*) normal inorganic phosphate in H_2O^{18} and (*b*) O^{18}-labeled inorganic

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[1] The following abbreviations will be used throughout this paper: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate; M_w, mitochondria; ADP, adenosine diphosphate.

phosphate in normal H_2O. Should the interaction of inorganic phosphate or of a phosphorylated intermediate with water in the medium be direct, no difference would be anticipated, whether the O^{18} is initially in the inorganic phosphate or in the water. On the other hand, if the time course of the replacement of oxygen differs under the two sets of conditions, the interaction with water must be indirect. It has been found that the rate of the inorganic phosphate-oxygen replacement reaction is essentially the same, irrespective of which reactant is initially labeled. On the other hand, in the transfer reaction to ATP the amount of O^{18} replaced in ATP does depend on which reactant is initially labeled.

Methods

Preparation of O^{18}-Labeled Phosphate—The inorganic phosphate containing 2.6 atom per cent excess was prepared as described previously (1) by reaction of P_2O_5 with H_2O^{18}. By this procedure, the O^{18} of the H_2O^{18} is diluted by a factor of 8/3 in the final product. Since it was desirable to avoid dilution, the inorganic phosphate with higher concentrations of O^{18} was prepared by exchange with water. A 2.8 M solution of KH_2PO_4 in H_2O^{18} was sealed in a tube which was kept at 120° for 8 days. Equilibration was practically complete, as anticipated from an extrapolation of the data of Blumenthal and Herbert (3). The water and KH_2PO_4 were separated by distillation *in vacuo*. The process may be repeated to obtain more labeled inorganic phosphate. The inorganic phosphate containing about 5 atom per cent excess was prepared in this way with water generously supplied by Dr. D. Rittenberg.

Materials—The ATP was obtained from the Pabst Brewing Company; the DPN, approximately 90 per cent pure, and cytochrome *c* were obtained from the Sigma Chemical Company. *dl*-β-Hydroxybutyric acid was a product of the Mallinckrodt Chemical Works. The yeast hexokinase was kindly supplied by Dr. R. K. Crane. The intestinal phosphatase used in these experiments was a commercial preparation of the Armour Laboratories and contained 15 units per mg. (4).

Analysis of Phosphate for O^{18}—The method described previously (1), decomposition of KH_2PO_4 to water with subsequent equlibration of the water with CO_2, required approximately 300 μmoles of phosphate and necessitated either experiments on a very large scale or rather large dilutions of the isotope concentration of the isolated phosphates. A new method was therefore devised in which 10 to 20 μmoles of phosphate could be analyzed for its O^{18} content. The method is based on the reaction of $Ba_3(PO_4)_2$ with carbon at approximately 1350° *in vacuo* to yield CO, which is analyzed directly in the mass spectrometer for the ratio of mass 30 to mass 28. The reduction of $Ca_3(PO_4)_2$ by carbon has been studied in some

detail (5), but the barium rather than the calcium salt was chosen because of the tendency of calcium to form basic salts and the consequent difficulty of preparing pure $Ca_3(PO_4)_2$.

The final $Ba_3(PO_4)_2$ was prepared from an acid solution of a barium phosphate precipitate by the addition, with stirring, of CO_2-free NaOH until the solution was alkaline to brom thymol blue. The covered tubes were allowed to stand in the cold room for $\frac{1}{2}$ hour to insure complete precipitation. The precipitate was then centrifuged and washed twice with 0.5 ml. portions of water. The tubes were kept covered during centrifuga-

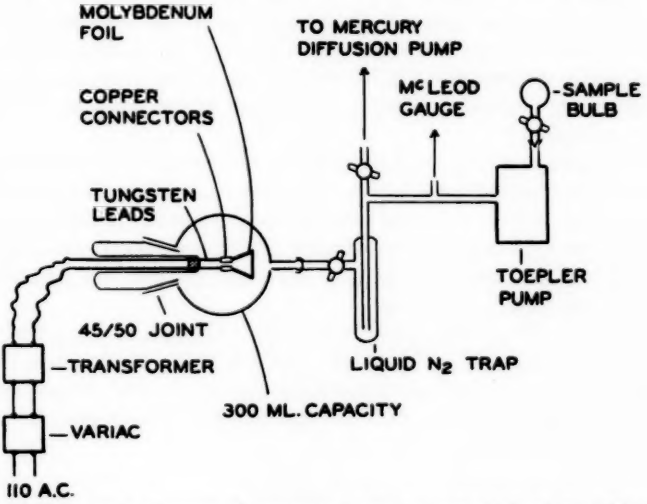

FIG. 1. Schematic diagram of apparatus for the reduction of $Ba_3(PO_4)_2$ with carbon.

tion to prevent precipitation of carbonate. The $Ba_3(PO_4)_2$ was dried for $\frac{1}{2}$ hour in an oven at 100° and then for 1 hour *in vacuo* in a pistol drier over P_2O_5 at 100°.

The carbon used for the reduction was powdered from a spectroscopic carbon rod obtained from the Jarrell-Ash Company. Before use, the carbon was degassed by pumping with a mercury diffusion pump and heating for 5 minutes at 800°, 10 minutes at 1000°, and 10 minutes at 1400°. The carbon was cooled and exposed to an atmosphere of helium gas. This procedure was carried out in the same apparatus used for the reduction shown in Fig. 1.

The apparatus is similar to the one employed by Hoering (6) for the analysis of O^{18} in sulfate by the reduction of $BaSO_4$ with carbon. The

solid $Ba_3(PO_4)_2$, approximately 6 mg., is mixed with 5 mg. of carbon and enclosed by folding over twice in a molybdenum foil, 1 cm. by 7 cm., 1 mil thick. The foil is clamped into connectors between two electrodes and forms a filament inside the reaction vessel (Fig. 1). The system is then evacuated until the pressure is approximately 1×10^{-4} mm. of Hg. Pumping is continued while the sample is heated for 10 minutes at 800° and for 5 minutes at 1000°. The reaction vessel is then closed off from the system and the filament heated to 1350° for 5 minutes. The reaction flask is cooled with water during the reaction to prevent the exchange of oxygen in the glass with any gas formed. The gas formed is then transferred by a Toepler pump to a sample bulb for analysis in the mass spectrometer. The temperature of the filament is controlled by varying the voltage on the input of the transformer which supplies current to the filament. The temperature at various voltages was calibrated with a Leeds and Northrup optical pyrometer.[2]

The advantage of this method over the previous one is the small amount of phosphate required for analysis. The chief disadvantage is the nature of the product, CO, a gas which has the same mass as N_2 and which cannot easily be freed of any contaminating N_2 for mass spectrometric analysis. The results obtained routinely with the method are reproducible within approximately 5 per cent.

Analysis for P^{32}—Carrier-free P^{32} inorganic phosphate was purified before use by heating at 100° for 1 hour in 1 N HCl. It was then treated with Norit to remove any adsorbable radioactivity. The radioactivity of all samples was measured with an end window Geiger counter and a standard scaler circuit.

EXPERIMENTAL

The mitochondria were prepared from rat livers in isotonic sucrose essentially as described previously (1). In the experiments on oxidative phosphorylation, the reaction mixture was placed in a stoppered 125 ml. Erlenmeyer flask and shaken in a water bath at the temperatures indicated in Tables I to III with air as the gas phase. For the inorganic P^{32}-ATP exchange reaction, the reaction mixture was incubated at room temperature in a centrifuge tube without shaking with air as the gas phase. Appropriate aliquots were withdrawn at given times to yield enough ATP or glucose-6-phosphate for isotopic analysis, and the reaction was stopped by the addition of sufficient ice-cold 20 per cent trichloro-

[2] The operation of the reduction apparatus and all handling and cleaning of the reaction vessels are carried out in a good hood, because appreciable amounts of phosphine are liberated when the products of the reduction of the phosphate come into contact with water.

acetic acid to bring the final concentration to 5 per cent. The inorganic phosphate and 10 minute-hydrolyzable phosphate were determined by the Fiske-Subbarow method (7).

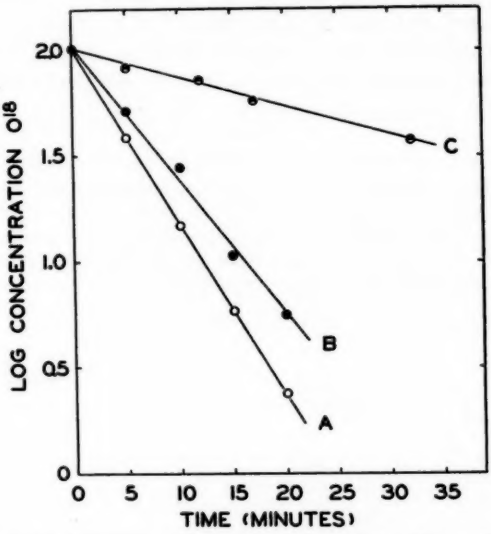

FIG. 2. Rate of replacement of oxygen in inorganic phosphate. Curves A and B are oxidative phosphorylation experiments described in Table III with $HPO_4^{16=}$ in H_2O^{16} and $HPO_4^{16=}$ in H_2O^{18}, respectively. Curve C represents an exchange experiment between P_i^{32} and ATP with $HPO_4^{16=}$ in H_2O^{18}. For Curves B and C, log $(100 - O^{18}_{P_i})$ is plotted *versus* time to make them comparable to Curve A. The reaction mixture for the experiment in Curve C consisted of 0.022 M P_i, 0.008 M ATP, 0.01 M $MgCl_2$, M_w per ml. equivalent to 0.25 gm. of fresh liver, initial H_2O^{18}, 1.09 atom per cent excess, initial P^{32}, 2426 c.p.m. per μmole of P_i. The rate of phosphate transfer calculated from the P^{32} exchange was 0.15 μmole per ml. per minute.

Isolation of Phosphate for Isotopic Analysis

Inorganic Phosphate—Inorganic phosphate was isolated as described previously (1) in the experiments in which adenylic acid was used as acceptor (Tables I and II) and in the exchange experiments (Fig. 2). In the experiments in Table I, the phosphate was finally precipitated as KH_2PO_4 (1) and diluted to measure the O^{18} in the water formed by dehydration; in all other experiments, the O^{18} was measured in CO by reduction of $Ba_3(PO_4)_2$ with carbon. When glucose was used as the acceptor (Tables I and III), the inorganic phosphate had to be separated from glucose-6-phosphate. This was accomplished by precipitating in the usual

way with barium acetate, dissolving the precipitate in acid, and removing the barium with K_2SO_4. The inorganic phosphate was then precipitated with magnesia mixture and the magnesium ammonium phosphate was allowed to crystallize overnight in the cold room. The precipitate was then centrifuged and dissolved in acid. The magnesium was removed either by precipitation with a large excess of potassium fluoride or with Dowex 50. Analysis for inorganic phosphate and total phosphate indicated that the inorganic phosphate was free of contamination. The phosphate was then precipitated as the $Ba_3(PO_4)_2$ for reduction with carbon.

TABLE I

Conversion of Inorganic Phosphate to ATP and Glucose-6-phosphate

Reaction mixture: Tris buffer 0.02 M, pH 7.4; phosphate 0.02 M; $MgCl_2$ 0.005 M; adenylic acid 0.008 M; cytochrome *c* 1×10^{-5} M; DPN 3×10^{-4} M; *dl*-β-hydroxybutyrate 0.02 M; liver mitochondria from 0.1 gm. of fresh liver per ml. of reaction mixture; $T = 25°$. In Series B, glucose, 0.025 M, and hexokinase were added. Initial atom per cent excess O^{18} in inorganic phosphate 2.60.

Series	Time	$-\Delta P_i$	P_i			ATP		
			O^{18}, relative concentration	Loss of O^{18}	*N*	O^{18}, relative concentration		Additional loss of O^{18}
						Observed $\times \frac{4}{3}$	Calculated	
	min.	*μmoles per ml.*		*per cent*				*per cent*
A	0		100					
	5	2.8	90.1	9.9	0.41 ± 0.4	34.1	95.2	64.2
	20	4.5	52.6	47.4	2.56	28.7	74.0	61.2
	60	3.9	21.4	78.6	6.25	23.1	50.4	54.1
						Glucose-6-phosphate		
B	0		100					
	5	3.2	82.0	18.0	0.79			
	20	6.6	47.5	52.5	2.98	20.4	70.5	71.1
	60	8.1	35.2	64.8	4.20	15.2	58.0	73.8

Isolation of Phosphate from ATP—The ATP was removed from the trichloroacetic acid supernatant fluid with Norit (8), as previously described. After being washed with 2 ml. portions of water to remove most of the labeled inorganic phosphate, the Norit was resuspended in 10 ml. of 3 per cent trichloroacetic acid containing 100 μmoles of inorganic phosphate to wash out any remaining labeled inorganic phosphate. After centrifugation, the Norit was washed three times with 10 ml. portions of water and then suspended in 2.0 ml. of 2 N HCl and heated with occasional stirring for 10 minutes at 100°. The inorganic phosphate formed in the hydrolysis

was then isolated in the usual way either as KH_2PO_4 (Table I) or as $Ba_3(PO_4)_2$ (Table II).

Isolation of Phosphate from Glucose-6-phosphate—The glucose-6-phosphate was precipitated as the barium salt by the addition of 4 volumes of 95 per cent ethyl alcohol to the supernatant fluid from the barium precipitation of inorganic phosphate. The precipitate was centrifuged, dissolved in 5 per cent trichloroacetic acid, and treated with Norit to remove adenylic acid. To the supernatant solution and washings from the Norit,

TABLE II

Oxidative Phosphorylation with Adenylic Acid As Acceptor; Series A, PO_4^{18} in H_2O^{16}; Series B, PO_4^{16} in H_2O^{18}

Reaction mixture: phosphate 0.02 M, pH 7.4; $MgCl_2$ 0.02 M; adenylic acid 0.007 M; *dl*-β-hydroxybutyrate 0.01 M; cytochrome *c* 3×10^{-5} M; DPN 7×10^{-4} M; KCl 0.075 M; M_w equivalent to 0.25 gm. of fresh liver per ml. of reaction mixture. Initial atom per cent excess O^{18}: Series A in inorganic phosphate, 5.17; Series B in H_2O, 0.500.

Series	Time	$-\Delta P_i$	P_i			ATP		
			O^{18}, relative concentration	Loss of O^{18}	*N*	O^{18}, relative concentration		Additional loss of O^{18}
						Observed × $\frac{3}{4}$	Calculated	
	min.	*μmoles per ml.*		*per cent*				*per cent*
A	7	3.4	80.0	20.0	0.89	21.4	89.0	76.0
	30	7.7	14.1	85.9	7.85	13.9	43.8	68.3
				Gain of O^{18}				Additional gain of O^{18}
B	7	3.8	26.0	26.0	1.2	58.8	13.3	52.5
	30	8.0	84.7	84.7	7.45	68.2	45.5	41.6

50 μmoles of ordinary inorganic phosphate were added, the pH was adjusted to 8.2, and barium acetate was added to precipitate the inorganic phosphate. After removal of the precipitate, the barium salt of glucose-6-phosphate was precipitated by the addition of 4 volumes of 95 per cent ethyl alcohol. The precipitate was centrifuged and dissolved in acid and Ba^{++} was removed with K_2SO_4.

The solutions containing glucose-6-phosphate were adjusted to pH 9.0 with NH_4OH, and 0.7 ml. of the magnesia mixture was added to remove any traces of inorganic phosphate. The solutions were allowed to stand in the cold room for several hours and any precipitate which formed was discarded after centrifugation. The solutions were adjusted to a final volume of 10 ml. and 5 mg. of Armour's intestinal phosphatase were added. The hydrolysis was allowed to proceed for 2 hours at 37° with occasional

stirring. To insure complete precipitation of the inorganic phosphate formed in the hydrolysis, 0.5 ml. of magnesia mixture was added at the end of the reaction and the solution was allowed to stand in the cold room overnight. The solution was centrifuged and the precipitate suspended in 5 per cent trichloroacetic acid. The $MgNH_4PO_4$ was dissolved and the

TABLE III

Oxidative Phosphorylation with Glucose As Acceptor; Series A, PO_4^{18} in H_2O^{16} and Series B, PO_4^{16} in H_2O^{18}

Reaction mixture: phosphate 0.02 M, pH 7.4; $MgCl_2$ 0.02 M; adenylic acid 0.00167 M; *dl*-β-hydroxybutyrate 0.01 M; cytochrome *c* 3×10^{-5} M; DPN 3×10^{-4} M; glucose 0.033 M; 0.1 ml. of hexokinase (sufficient to transfer 15 μmoles per ml. of reaction mixture from ATP in 5 minutes); KCl 0.075 M; M_w equivalent to 0.2 gm. of fresh liver per ml. of reaction mixture; $T = 30°$. Initial atom per cent excess O^{18}: Series A in inorganic phosphate 5.00; Series B in H_2O, 0.628.

Series	Time	$-\Delta P_i$	P_i			Glucose-6-phosphate		
			O^{18}, relative concentration	Loss of O^{18}	N	O^{18}, relative concentration		Additional loss of O^{18}
						Observed × ½	Calculated	
	min.	*μmoles per ml.*		*per cent*				*per cent*
A	0		100					
	5	3.2	38.2	61.8	3.85	21.2	64.3	67.0
	10	7.1	14.7	85.3	7.72	14.4	44.2	67.5
	15	10.0	5.85	94.1	11.4	12.5	33.2	62.3
	20	12.5	2.40	97.6	14.9	10.2	26.2	61.1
				Gain of O^{18}				Additional gain of O^{18}
B	0		0					
	5	4.9	49.8	49.8	2.76	39.6	27.8	16.3
	10	8.4	72.0	72.0	5.08	63.4	43.3	35.4
	15	10.8	89.4	89.4	8.96	77.0	60.1	42.4
	20	12.8	94.5	94.5	11.6	86.0	67.4	57.0

insoluble residue of phosphatase was discarded after centrifugation. The acid solution of $MgNH_4PO_4$ was treated as described for the inorganic phosphate fraction to obtain $Ba_3(PO_4)_2$ for O^{18} analysis.

Results

Methods of Calculation

Determination of N, Number of Cycles of Reaction of Inorganic Phosphate—In a previous paper (1), it was shown that the ratio of the final

concentration of O^{18} in the inorganic phosphate to the initial concentration may be expressed as $(\frac{3}{4})^N$, where N is the number of reaction cycles which the phosphate has undergone, one oxygen atom, one-fourth of the total O^{18}, being replaced in each cycle if, for example, a monoester of phosphate is formed and then hydrolyzed. In this formulation, it is assumed that all the phosphate reacts to lose one-fourth of its O^{18} in one cycle, followed by a second cycle in which all the phosphate reacts to lose a fourth of the O^{18} which remains, and so on. A more exact formulation is based on continuous cycling rather than on discrete steps and is given by the following expression, where F is the ratio of O^{18} concentration at time t to the initial O^{18} concentration:[3]

$$F = \frac{[O^{18}]_{\text{at time } t}}{[O^{18}]_{\text{initial}}} = [e^{-\frac{1}{4}}]^N = [\tfrac{3}{4} + \tfrac{1}{3!2} + \ldots]^N = [0.799]^N \qquad (1)$$

For experiments in H_2O^{18}, the initial concentration in the phosphate is zero; an equivalent expression for the evaluation of N may be obtained by using the O^{18} concentration in phosphate at equilibrium, *i.e.* the O^{18} concentration in the water, and the formulation becomes

$$F = \frac{[O^{18}]_{\text{equilibrium}} - [O^{18}]_{\text{at time } t}}{[O^{18}]_{\text{equilibrium}}} = 1 - \frac{[O^{18}]_t}{[O^{18}]_{\text{equilibrium}}} = [e^{-\frac{1}{4}}]^N \qquad (2)$$

Calculated Value of O^{18} in Phosphate of ATP or Glucose-6-phosphate—The expected O^{18} concentration in the phosphate of ATP or glucose-6-phosphate is calculated on the assumption that the O^{18} concentration of the ATP formed at any instant is equal to the O^{18} concentration of the inorganic phosphate of the medium. This value at any time t is equal to the average value in the inorganic phosphate from which the ATP has been formed during the interval from time $t = 0$ to time t, and is given by the expression

$$F_{0 \to t} = \frac{1}{N} \int_0^N e^{-\frac{1}{4}N}\, dN = \frac{1}{\frac{1}{4}N} [1 - e^{-\frac{1}{4}N}] \qquad (3)$$

The validity of this expression as a time average is established by the experimental finding that N (Equation 1) is a linear function of time. Since the initial concentration of O^{18} in each experiment is arbitrarily set at 100 to make all experiments comparable, the average value of the O^{18} concen-

[3] The relation of N to the rate constant may be shown by the following considerations. The over-all reaction of the replacement of oxygen in inorganic phosphate may be represented as an exchange with water: $H_3PO_4{}^{18} + H_2O^{16} \xrightarrow{k} H_3PO_3{}^{18}O^{16} + H_2O^{18}$; $(d[O^{18}{}_{H_3PO_4}]/dt) = -\frac{1}{4}k[O^{18}{}_{H_3PO_4}]$, since only one-fourth of the O^{18} in H_3PO_4 is lost in this reaction. The integrated form of the rate equation is $([O^{18}{}_{H_3PO_4}]$ at time $t/[O^{18}{}_{H_3PO_4}]_{\text{initial}}) = e^{\frac{1}{4}kt}$. Therefore N (Equation 1) $= kt$.

tration in inorganic phosphate relative to the initial value in the interval from the initial time to time t is $100 \times F_{0 \rightarrow t}$ as defined in Equations 1 and 3. Similarly, for experiments in H_2O^{18},

$$F_{0 \rightarrow t} = \frac{1}{\frac{1}{4}N}[1 - e^{-\frac{1}{4}N}] = 1 - \frac{[O^{18}]_{average}}{[O^{18}]_{equilibrium}} \quad (4)$$

and if O^{18} equilibrium, *i.e.* the O^{18} concentration of the water, is set at 100, the average value of the O^{18} concentration in inorganic phosphate relative to the initial value in the interval from the initial time to time t is $100\ (1 - F_{0 \rightarrow t})$.

O^{18} Concentration in ATP and Glucose-6-phosphate—In the inorganic phosphate prepared for isotopic analysis from ATP and glucose-6-phosphate by acid hydrolysis and hydrolysis by phosphatase, respectively, one of the four oxygens is introduced from the normal water of the hydrolysis medium. The observed value of the O^{18} must therefore be multiplied by $\frac{4}{3}$ to give the value of O^{18} in the three oxygens which have been derived from the phosphate groups of ATP and glucose-6-phosphate.

Per Cent Additional Loss or Gain of O^{18} in ATP or Glucose-6-phosphate—To calculate the percentage of oxygen which has been replaced in ATP beyond that which has necessarily been replaced by way of the inorganic phosphate, the average value of O^{18} in the inorganic phosphate from which the ATP has been derived is taken as the base-line. This value has been called the calculated value of ATP (Equations 3 and 4) and is considered the initial value of O^{18} in ATP for calculating the additional replacement for the experimentally observed value of O^{18} in ATP. The additional per cent loss in the case of initially labeled phosphate is thus defined as

$$\frac{ATP_{calculated} - ATP_{observed}}{ATP_{calculated}} \times 100 \quad (5)$$

For H_2O^{18}, the analogous additional per cent gain is defined as

$$\frac{(100 - ATP_{calculated}) - (100 - ATP_{observed})}{(100 - ATP_{calculated})} \times 100 = \frac{ATP_{observed} - ATP_{calculated}}{100 - ATP_{calculated}} \times 100 \quad (6)$$

Equations 5 and 6 are equivalent to $(1 - F_{ATP}) \times 100$, where F_{ATP} is analogous to F_{P_i} defined in Equations 1 and 2, respectively.

Conversion of Inorganic Phosphate-O^{18} to ATP and Glucose-6-phosphate—The results of an experiment on oxidative phosphorylation with *dl*-β-hydroxybutyrate as substrate and labeled inorganic phosphate are given in Table I. Adenylic acid was the phosphate acceptor in Series A and glucose

was the acceptor in Series B. The O^{18} concentrations listed are relative to the initial concentration, 2.60 atom per cent excess, which was set at 100; *i.e.*, all experimentally determined values have been multiplied by 100/2.6. The values for N, $ATP_{calculated}$ or $glucose\text{-}6\text{-}phosphate_{calculated}$, and the per cent additional loss in ATP or glucose-6-phosphate have been calculated according to Equations 1, 3, and 5, respectively. It will be noted from Table I that the O^{18} concentration observed in ATP or glucose-6-phosphate is considerably lower than the calculated value. At short time periods, the observed value of O^{18} in ATP or glucose-6-phosphate is even lower than the value of O^{18} in inorganic phosphate at the end of the period. The ratio of the observed value to the calculated value in the product of phosphorylation, ATP or glucose-6-phosphate, is about one-third in each case.

Although this experiment clearly shows the difference between oxygen replacement in inorganic phosphate and ATP or glucose-6-phosphate, it was quantitatively unsatisfactory as a rate study for several reasons. The uptake of inorganic phosphate was not linear with time, and the rate of ATP breakdown became large relative to its rate of formation after 20 minutes. The ATPase activity leads to a significant lowering of the O^{18} in inorganic phosphate by the hydrolysis of ATP, which is superimposed on the loss of O^{18} in inorganic phosphate due to the inorganic phosphate-oxygen replacement reaction associated with oxidative phosphorylation. This effect is reflected in the calculated value for ATP or glucose-6-phosphate and obscures the quantitative relationship between the anticipated and experimentally observed values. Secondly, under the conditions of the experiment, the return of phosphate from ATP to inorganic phosphate by reversal (2) is undoubtedly occurring to some extent and masking the quantitative relations involved in the direction from inorganic phosphate to ATP. Lastly, the error of the analytical method, ± 10 per cent, leads to a large error in the evaluation of N, particularly at short times.

For the reasons discussed, the experiment was repeated when a better analytical method was available, and a 5-fold excess of hexokinase was added in an attempt to avoid the complications of ATPase and the reversal of phosphorylation. The results are presented in Series A of Table III. It will be noted that the phosphate uptake (ΔP_i) is fairly linear with time and the decrease of O^{18} concentration in inorganic phosphate is exponential with time (Fig. 2), and consequently the number of cycles of reaction which the inorganic phosphate has undergone, N, is linear with time. The overall phosphorylation exhibits zero order kinetics and the system is saturated with respect to inorganic phosphate, but the inorganic phosphate-oxygen replacement reaction exhibits first order kinetics (Fig. 2) and the system is not saturated even at a phosphate level of 17 μmoles per ml. Obviously,

the inorganic phosphate-oxygen replacement reaction is not the rate-limiting step in the sequence of reactions leading to the formation of ATP. As pointed out in a previous paper (1), the number of phosphate molecules which have had one oxygen atom replaced ($P_i \times N$) can greatly exceed the number of phosphate molecules transferred (ΔP_i); for example, in the first 5 minutes the O^{18} replacement reaction involves approximately 70 μmoles compared to 3 μmoles of phosphate transferred. This ratio would be meaningful only when saturation is reached for the replacement reaction as well as for the phosphorylation system.

In agreement with the data presented in Table I, the concentration of O^{18} in the glucose-6-phosphate is much lower than was anticipated from the average value in the inorganic phosphate from which it is formed. The ratio of the observed to the calculated value of the O^{18} in glucose-6-phosphate is practically constant, 0.30:0.39.

Comparison of Inorganic Phosphate-O^{18} in Water-O^{16} and Inorganic Phosphate-O^{16} in Water-O^{18}—A comparison of the two experiments in Table II indicates that in the oxygen replacement in inorganic phosphate there is no essential difference, whether the O^{18} is initially in the phosphate or in the water. For the ATP, qualitatively the results are the same in Series A and Series B; there was an additional loss of O^{18} in H_2O^{16} and an additional gain in H_2O^{18}. Quantitatively, it appeared that less oxygen was replaced when the medium was H_2O^{18} than when it was H_2O^{16}. Definite interpretation of the data, however, was limited by the difficulties with adenylic acid as acceptor, already discussed (Table I). For a more quantitative evaluation, the experiment was repeated with glucose as the acceptor, and more frequent samples were taken as shown in Table III. For Series B (H_2O^{18}), just as in Series A (H_2O^{16}), the number of cycles of reaction or inorganic phosphate, N, is a linear function of time, although the absolute rate is somewhat lower in Series B (Fig. 2). The striking difference lies in the values for glucose-6-phosphate, *i.e.* in the percentage of O^{18} lost beyond the anticipated value in Series A (Equation 5) and the percentage of O^{18} gained beyond the anticipated value in Series B (Equation 6). In Series A, the additional loss is constant with time, and with Series B, the additional gain increases from 16 to 57 per cent with time and approaches the value of Series A. It should be pointed out that the slow rise of the additional gain in Series B with time is somewhat misleading since the O^{18} found in glucose-6-phosphate at any time is the cumulative value of the glucose-6-phosphate from zero time and includes the glucose-6-phosphate formed in the first few minutes. If the latter is low in O^{18}, then its contribution continues in every subsequent time period and the gain in O^{18} can never reach the value for the loss in Series A which is apparently constant from the beginning.

Comparison of Oxidative Phosphorylation and Phosphate (P^{32})-ATP Ex-

change Reaction—In Fig. 2, it is shown that the O^{18} concentration in inorganic phosphate is a logarithmic function of time; *i.e.*, N, the number of cycles of reaction, is linear with time both in oxidative phosphorylation and in the exchange reaction. Curves A and B represent the data presented in Table III for Series A and B, respectively. Curve C represents an experiment with no net phosphorylation, an exchange between inorganic phosphate (P^{32}) and ATP without any substrate in a medium containing H_2O^{18}. The rate of phosphate transfer as measured by the exchange of P^{32} between inorganic phosphate and ATP was approximately 20 to 25 per cent of the rate of phosphate transfer of the oxidative phosphorylation Experiments A and B, as measured by the uptake of inorganic phosphate. This difference in rate of phosphate transfer is reflected in the difference in the rate of O^{18} replacement in inorganic phosphate shown in Fig. 2.

DISCUSSION

It has been suggested by several investigators (9, 10), on the basis of studies on the uncoupling of oxidation and phosphorylation by 2,4-dinitrophenol, that the sequence of reactions from inorganic phosphate to ATP in oxidative phosphorylation involves several intermediate steps. The difference in the results obtained in the present study with O^{18}-labeled inorganic phosphate and O^{18}-labeled water, respectively, strongly supports this concept. The rate of replacement of oxygen in inorganic phosphate and in ATP would be similarly affected by the initial position of the isotope in any mechanism postulating only a single intermediate between inorganic phosphate and ATP. However, it can be seen from the data (Table III) that the rate of replacement of oxygen in the inorganic phosphate is a similar function of time in H_2O^{16} or H_2O^{18}, but the additional replacement of oxygen in the ATP remains essentially constant in H_2O^{16}, while it varies considerably with time in H_2O^{18}. It would be difficult to reconcile these results with a mechanism postulating a single intermediate between inorganic phosphate and ATP.

Thus, the results of the present study indicate that there are at least two kinetically distinguishable steps in the formation of ATP from inorganic phosphate coupled to electron transport. It has not yet been possible to demonstrate the independent existence of a second reaction directly. In this discussion it is assumed that these steps represent chemical reactions. However, the possibility that the behavior is due to a segregation of activity into different regions in the mitchondria cannot be excluded. To explain all the observed facts would entail a rather complex segregation of this kind, including separate phosphate and water compartments within the mitochondria. The nature of these divisions, whether physical or chemical, requires further investigation.

Boyer and coworkers (2) have postulated a one-step mechanism on the

basis of data obtained on the oyxgen replacement in inorganic phosphate in a mitochondrial system which is exhibiting exchange of inorganic phosphate (P^{32}) and ATP with no net change in either component. The mechanism suggested by these authors introduces the oxygen from the water into inorganic phosphate by a rapid reversal of the first step coupled to the electron transport system in which organic phosphate is formed; namely, $HPO_4^= + YH \rightleftharpoons YPO_3^= + H_2O$. The phosphate is then transferred from $YPO_3^=$ to ADP. This mechanism is adequate to explain the data obtained by Boyer *et al.* (2), and would also suffice to explain the replacement of oxygen in inorganic phosphate in our experiments. However, it is insufficient to account for the observations on ATP. By this mechanism, no difference would be expected in experiments with inorganic phosphate-O^{18} and H_2O^{18}. Moreover, it does not account for our finding that much more oxygen has been replaced in the ATP than in inorganic phosphate. It follows from the above mechanism that the concentration of O^{18} in inorganic phosphate arising from H_2O^{18} must be at least as high as that in ATP, because the O^{18} can be introduced into ATP only by first entering the inorganic phosphate. It should be pointed out that we have investigated the inorganic phosphate-ATP exchange reaction with O^{18} and have found that, as in oxidative phosphorylation, much more oxygen is replaced in the phosphate transferred to ATP than in inorganic phosphate. The details of these experiments will be reported later.

Let us now consider, in the light of a multistep mechanism of phosphorylation, the observation that the oxygens of the phosphate of ATP synthesized in oxidative phosphorylation have undergone more replacement from the water of the medium than have those of the inorganic phosphate from which they have been ultimately derived. The question arises as to how much of the oxygen which has been replaced in the ATP is accounted for by the first reaction which is responsible for the replacement of oxygen in inorganic phosphate, and how much is due to further replacement in a subsequent reaction or in reactions leading to the formation of ATP. A quantitative interpretation of the data is complicated by the uncertainty in equating the observed concentration of the isotope in the inorganic phosphate with that of the intermediate formed in the first reaction. It might be argued that there is a small pool of inorganic phosphate or of the first intermediate which exchanges oxygen very rapidly with the water of the medium and which more slowly exchanges phosphate with the large experimentally observed phosphate pool. The most cogent evidence in favor of the existence of isotopic equilibrium between the experimentally determined inorganic phosphate and the first intermediate is obtained from the 5 minute experiment in H_2O^{18} (see Series B, Table III) in which the observed value of the O^{18} in ATP is only 16 per cent above the average

value in the inorganic phosphate of the medium ($ATP_{calculated}$). This can only mean that the maximal difference in isotopic composition between the inorganic phosphate of the medium and the first intermediate is 16 per cent. The amount of oxygen replaced beyond the first step of the phosphorylation sequence can be estimated. The finding that the O^{18} concentration in ATP is only one-third (see Series A, Table III) of that anticipated from the average value in the O^{18}-labeled inorganic phosphate may be interpreted to mean that approximately two of the three oxygens in the transferred phosphate have been replaced beyond the step in which the inorganic phosphate has lost its labeled oxygen.

The difference in behavior of the oxygen replacement in ATP when the O^{18} is initially in the water rather than in the phosphate suggests that, beyond the first step in the phosphorylation sequence, an intermediate other than water is involved which supplies its oxygen to the phosphate of ATP. Eventually the oxygen of water replaces the oxygen in this intermediate. When the reaction proceeds in H_2O^{16}, the oxygen in the intermediate is indistinguishable from that of the water at all times. On the other hand, when the reaction proceeds in H_2O^{18}, the oxygen of the intermediate which is initially unlabeled can be distinguished from the oxygen of water early in the reaction.

In the foregoing discussion, it has been tacitly assumed that there exists a similar or common pathway of inorganic phosphate to ATP at each step in the electron transport system. Evidence from earlier work (1) suggested that the oxygen replacement reaction in inorganic phosphate was not limited to a single step; the question is now being reinvestigated for the oxygen replacement in inorganic phosphate and ATP. The recent demonstration (11, 12) that phosphorylation, coupled to the oxidation of cytochrome *c* with oxygen, can be experimentally observed makes possible a study with O^{18} of the phosphorylation in this region alone.

In addition to the possibility that such an approach may provide a method of distinguishing the pathways of phosphorylation at different steps in the electron transport system, it may also throw some light on the nature of the reversal of phosphorylation. Our experimental results indicate a quantitative difference in the amount of O^{18} which appears in ATP in oxidative phosphorylation and in the reversible reaction between inorganic phosphate and ATP; more oxygen is replaced in ATP per phosphate transferred in the reversible reaction than in oxidative phosphorylation. This difference can be accounted for on the basis of differences in rates of the various reactions involved. On the other hand, it can also be accounted for if one postulates that there are different pathways in the conversion of inorganic phosphate to ATP at the different steps in the electron transport system, some involving additional replacement of oxy-

gen in ATP, others not. The consequence would be that what is observed in ATP in oxidative phosphorylation is an average due to different mechanisms. If the P^{32} exchange, *i.e.* the reversible reaction, occurred only at some steps, namely the steps in which the mechanism of transfer involved a very rapid replacement of oxygen in ATP, then the observed higher replacement in the reversible reaction could be explained. This hypothesis is now being tested.

SUMMARY

The formation of ATP from inorganic phosphate in oxidative phosphorylation associated with the electron transport system in liver mitochondria was investigated with O^{18} and the following results were revealed.

1. When oxidative phosphorylation is studied with O^{18}-labeled inorganic phosphate, the isotope concentration falls more rapidly in ATP than in inorganic phosphate. Similarly, with O^{18}-labeled water, the isotope concentration rises more rapidly in ATP than in inorganic phosphate.

2. The rate of replacement of oxygen in inorganic phosphate is exponential with time, both in oxidative phosphorylation and in the exchange reaction between inorganic phosphate and ATP.

3. A comparison of the reaction of $HPO_4^{18=}$ in H_2O^{16} and $HPO_4^{16=}$ in H_2O^{18} showed that the rates were essentially the same for the replacement of oxygen in inorganic phosphate but different for the replacement of oxygen in ATP. The implications of these findings for the mechanism of phosphorylation associated with oxidation in this system are discussed.

A new method of analysis of O^{18} in inorganic phosphate is described.

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ISOLATION OF DEOXYCHOLIC ACID FROM NORMAL HUMAN FECES*

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The isolation of deoxycholic acid from human feces was described by Fischer in 1911 (1). This finding, however, appears to have been largely overlooked, as in subsequent studies on the excretion of bile acids in human feces methods have been employed by which only cholic acid is measured, giving no information concerning the presence of deoxycholic acid (2–4). The application to normal human feces of chromatographic techniques for separating bile acids (5–7) has resulted in the isolation of crystalline deoxycholic acid, thus confirming Fischer's observation and also demonstrating that this is the principal bile acid in the feces of healthy adult humans. The details of the procedure and the methods used for identification are described in the following.

Methods

Extraction of Feces—800 gm. of feces, collected over a 5 day period from a healthy adult male and stored at −18°, were homogenized with 2 liters of boiling ethanol and filtered through a large Büchner funnel. The residue was extracted three more times in the same manner. The final filtrate was almost colorless and the residue a pale yellow. The combined filtrates, totaling 8 liters, were concentrated under reduced pressure at 40° to 250 ml. of a dark brown syrupy mixture. This mixture was combined with 700 ml. of water and adjusted to pH 8 with 7 N sodium hydroxide. The alkaline solution was extracted with three 1 liter portions of petroleum ether (b.p. 30–68°). The aqueous phase was then slowly acidified to a blue reaction on Congo red paper, with hydrochloric acid, and extracted three times with 1 liter portions of reagent grade chloroform. The combined chloroform extracts were concentrated to dryness under reduced pressure. The residue was rinsed from the flask with acetone. This was kept overnight in a desiccator containing P_2O_5. The resulting mixture consisted of a dark brown glue-like material measuring 10 ml.

Column Partition Chromatography—The residue from the chloroform ex-

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tract was mixed with 9 drops of glacial acetic acid and enough dry Celite to make a sticky powder and chromatographed on a Celite column (1.9 × 30 cm.) which utilized 70 per cent aqueous acetic acid as the stationary phase and petroleum ether-isopropyl ether as the moving phase. The preparation and operation of these columns have been described by Mosbach and coworkers (5).

Elution was started with 150 ml. of petroleum ether and continued with 200 ml. amounts of petroleum ether-isopropyl ether mixtures containing 40, 55, and 60 per cent isopropyl ether respectively. The 40 per cent isopropyl ether effluent was reduced to dryness and chromatographed a second time in the same manner and with the same sequence of solvents. The 40 per cent isopropyl ether fraction from this second column was evaporated, and the residue consisted of 300 mg. of a white residue contaminated with traces of pigment.

Crystallization—The residue from the 40 per cent isopropyl ether effluent was twice crystallized from glacial acetic acid, three times from ethyl acetate, and twice from aqueous ethanol. After drying over P_2O_5 at 26° and 7 mm. of Hg for 48 hours, the crystals weighed 31 mg. and melted at 173–174°. The melting point of the crystals was unaltered by admixture with an authentic sample of deoxycholic acid crystallized in a similar manner.

Preparation of Deoxycholic Acid Derivative—The stearic acid derivative of deoxycholic acid was prepared by dissolving 8 mg. of the crystalline substance from feces and 1 mg. of stearic acid in boiling ethanol. Water was then added to the point of turbidity and the mixture slowly cooled to 5°. The crystals obtained melted at 186° (8).

Infra-Red Spectrometry—The infra-red spectrum (9, 10) produced by 1 mg. of the crystalline material from feces was identical with that of an authentic sample of deoxycholic acid. The spectra were recorded through a frequency range between 4000 and 650 $cm.^{-1}$ by means of a Perkin-Elmer model 21 spectrometer and the potassium bromide disk method.[1]

Ultraviolet Absorption—A solution containing 0.1 mg. of the crystals from feces dissolved in 5 ml. of 65 per cent aqueous sulfuric acid was heated in a water bath at 60° for 15 minutes and then cooled in water at room temperature for 15 minutes (11). Approximately 3 ml. of the solution were transferred to a 1 cm. cuvette and the absorption spectra determined with a Beckman DU quartz spectrophotometer. The absorption maximum occurred as a sharp peak at 3850 A, which is characteristic for dihydroxycholanic acids (11).

Paper Chromatography and Antimony Trichloride Reaction—Whatman

[1] The spectrograms were made in the infra-red laboratory, Division of Physical Chemistry, through the courtesy of Professor Bryce Crawford. We are indebted to Miss Ruth Cardinal for technical assistance.

No. 1 paper was treated with 70 per cent aqueous acetic acid, and 0.06 mg. of the crystals from stool dissolved in acetone was applied. Similar amounts of authentic deoxycholic and its isomer, chenodeoxycholic acid, were applied separately as reference compounds. The developing solvent, petroleum ether (80 per cent)-isopropyl ether (20 per cent), was allowed to descend on the paper strip for 12 hours (12). The chromatograms were then treated with antimony trichloride and observed in ultraviolet light

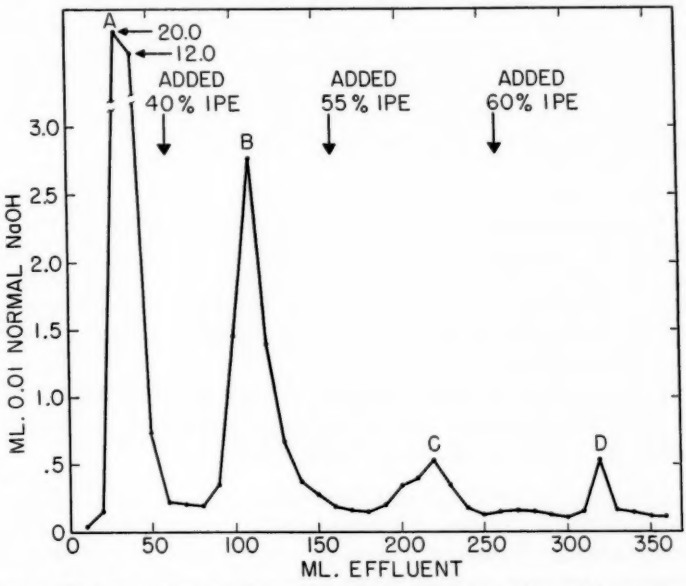

FIG. 1. Elution pattern of deoxycholic acid and certain other acidic components from normal human feces. *A*, fatty acids; *B*, deoxycholic acid; *C* and *D*, unidentified. IPE = isopropyl ether.

(13). The material isolated from feces moved at the same rate as deoxycholic acid and more rapidly than chenodeoxycholic acid. Its pink fluorescence was identical with that of deoxycholic acid and easily distinguished from the purple fluorescence of the more slowly moving isomer, chenodeoxycholic acid.

DISCUSSION

A simple modification of the above preparative procedure has been devised for determining the excretion of deoxycholic acid in feces of patients during studies of bile acid metabolism. For this purpose, 20 gm. of stool

are used with proportionately smaller volumes of solvents and a column measuring 1.2 × 30 cm. The amount of deoxycholic acid in the 40 per cent isopropyl ether eluent is determined by titration with 0.01 N sodium hydroxide. The resulting elution pattern of such a 20 gm. sample is illustrated in Fig. 1.

Among twenty-one normal subjects studied thus far the relative size of the various bands in the elution pattern has been quite consistent, showing that deoxycholic acid, as represented by the second band, is the principal bile acid excreted in the feces of healthy adults. The first band consists largely of fatty acids. Although the substances in the smaller third and fourth elution bands behave on the column as cholic acid and dehydrocholic acid, respectively, preliminary studies with paper chromatography have failed to reveal either of these compounds in the eluent.

SUMMARY

1. A chromatographic procedure for separating bile acids has been employed in the isolation of crystalline deoxycholic acid from an ethanolic extract of human feces. The identity of deoxycholic acid was established by means of melting point determinations, ultraviolet and infra-red spectrometry, paper chromatography, antimony trichloride reaction, and preparation of a deoxycholic acid derivative.

2. By adaptation to small amounts of feces the same procedure has proved useful and indicates that deoxycholic acid is the main bile acid excreted in the feces of normal adult human beings.

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ABSORPTION OF SUGARS IN VITRO BY THE INTESTINE OF THE GOLDEN HAMSTER*

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As early as 1900, Hédon (1) demonstrated that isotonic solutions of glucose and galactose were absorbed more rapidly than similar solutions of arabinose, an observation suggesting selective absorption of certain hexoses. This observation has been amply confirmed and at least two general groups of sugars delineated, one group of rapidly absorbed sugars (glucose and galactose) and another more slowly absorbed (mannose, sorbose, xylose, arabinose, and all disaccharides), with fructose intermediate between the two (2–4). The absorption of the first group is inhibited by the poisons iodoacetate (4) and phlorizin (5, 6), while that of the latter is unaffected. Furthermore, the absorption of glucose, galactose, and fructose (but not of other sugars) is associated with an accumulation of organic phosphates within the intestinal mucosa (7–9). On the basis of this and other evidence, it is generally accepted that only glucose, galactose, and fructose are absorbed by mechanisms mediated by enzyme reactions (probably phosphorylation and dephosphorylation) (4) and requiring the expenditure of energy.

Recently, however, a number of observations have complicated this generalization. Hele (10, 11) has shown that mannose and xylose are also phosphorylated by the intestinal mucosa; the rate of phosphorylation of these sugars *in vitro* parallels the rate of their absorption *in vivo*. Naito (12) has shown rapid absorption of ribose compared with other pentoses and accumulations of phosphate esters in the mucosa during its absorption. He concluded that ribose is phosphorylated and that it is absorbed by an active process. Kjerulf-Jensen (13) concluded that the fructose-1-phosphate which he isolated from intestines absorbing fructose was probably an intermediate not only in glucose formation but also in the active transport of fructose, as such, across the epithelium of the intestine.

It seemed desirable to reinvestigate the behavior of various sugars with one of the recently developed *in vitro* techniques (14). Two criteria for active transport may be tested with this method: (1) absorption against a concentration gradient, and (2) inhibition of transport when energy-yield-

* A preliminary communication was presented at the Forty-sixth annual meeting of the American Society of Biologial Chemists, San Francisco, 1955.

ing reactions are inhibited. This method has also facilitated the study of hydrolysis and absorption of disaccharides as well as the conversion of one sugar to another.

Methods

Preparation of Tissue—Unfasted male or female golden hamsters were killed by a blow on the head. The small intestine was washed out *in situ* with saline and removed from the animal. The intestine was then turned inside out with the aid of a probe and placed in a Petri dish containing saline at room temperature. Segments (3 to 4 cm. long) were carefully blotted and filled with a measured volume of bicarbonate-saline (15) from a small syringe and tied at both ends as described previously (14). This volume was taken as the initial serosal volume. The small sacs were then placed in 25 ml. Warburg flasks which contained 3.0 ml. of bicarbonate-saline (the initial mucosal volume). Flasks were gassed with either 5 per cent CO_2 and 95 per cent O_2 or 5 per cent CO_2 and 95 per cent N_2. Yellow phosphorus was placed in the center well in each anaerobic experiment to insure complete removal of oxygen. The flasks were incubated in a water bath for 1 hour at 37° with about 100 oscillations per minute. At the end of the incubation period the final serosal volume was determined by weighing the sac before and after emptying. The final mucosal volume was calculated from the difference between the initial mucosal volume and the gain or loss of fluid on the serosal side.

Solutions—The sugars were dissolved in bicarbonate-saline in all experiments except those with disaccharides and starch. 1 per cent solutions of disaccharides were prepared by mixing 1 part of 10 per cent sugar with 9 parts of bicarbonate-saline; 1 per cent starch was prepared by mixing equal parts of 2 per cent starch solution (made up in 0.9 per cent saline) and bicarbonate-saline. The following sugars were used: D(+)-glucose, 3-methyl-D(+)-glucose, D(+)-galactose, D(−)-fructose, L(−)-sorbose, D(+)-mannose, D(−)-ribose, D(+)-xylose, L(+)-arabinose, D(+)-maltose, D(+)-lactose, sucrose, soluble potato starch (Baker).

Analytical Methods—Reducing sugar and keto sugar were determined by the methods of Nelson (16) and Dische and Borenfreund (17), respectively. Descending paper chromatography (Whatman No. 1 paper) was used to separate sugars. The solvent in the sucrose experiments was *n*-butanol-acetic acid-water (4:1:5) (18); the solvent for all other experiments was *n*-butanol-pyridine-water (6:4:3) (19). A benzidine and trichloroacetic acid spray was used to develop the sugar spots (20). Quantitative estimation of sugars separated on paper chromatograms was carried out by cutting the sugar-containing area of filter paper (usually 5 × 2 cm.) into small strips and extracting in 5 ml. of water at room temperature for 20

minutes. Following centrifugation, aliquots were removed and reducing sugar or keto sugar was determined with appropriate paper blanks run simultaneously.

Units for Sugar Utilization or Transport—The quotients are similar to those used in a previous publication (14) and consist of the following: Q (sugar utilized) = microliters of sugar disappearing from the system per mg. dry weight per hour. Q (sugar movement) = microliters of sugar moving from one side of the intestinal wall to the other per mg. dry weight per hour. Q (fructose to glucose) = microliters of glucose formed from fructose per mg. dry weight per hour. Q (sugar hydrolyzed) = microliters of disaccharide hydrolyzed per mg. dry weight per hour. The sugars are expressed as microliters of gas (1 μmole = 22.4 μl.).

TABLE I

Absorption of Fructose by Hamster Intestine

Sac of everted jejunum (550 mg. wet weight) placed in bicarbonate-saline in 125 ml. Warburg flask and gassed with 5 per cent CO_2 and 95 per cent O_2. Incubation for 1 hour at 37°.

Sample	Fructose		Glucose		Volume
	Concentration	Amount	Concentration	Amount	
	mg. per 100 ml.	*mg.*	*mg. per 100 ml.*	*mg.*	*ml.*
Initial mucosal side	205	20.5	0	0	10.0
" serosal "	0	0	0	0	2.5
Final mucosal side	47	4.8	5.5	0.56	10.2
" serosal "	12	0.28	197	4.54	2.3

Results

Fructose—When a fructose solution was placed on the mucosal side of an everted sac of hamster intestine, the serosal solution was found after 1 hour's incubation to contain considerably more reducing sugar than keto sugar, indicating the presence of a second reducing sugar. The second sugar was identified as glucose by paper chromatography by using either the *n*-butanol-acetic acid-water solvent or the *n*-butanol-pyridine-water solvent. (Recently, the formation of glucose from fructose in preparations of guinea pig intestine *in vitro* has been identified with the use of glucose oxidase (21).) In one experiment (Table I) the concentration of glucose appearing on the serosal side was 36 times greater than that on the mucosal side. At the same time the fructose concentration on the serosal side was only one-fourth that on the mucosal side.

Fig. 1 shows the final concentrations of the two sugars when the same concentration of fructose was placed initially on both sides of the intestine.

Fructose did not move across the wall against a gradient but decreased in concentration on both sides to the same extent. A considerable portion of the fructose which disappeared is converted to glucose which appeared in higher concentration on the serosal side. When fructose was placed on the serosal side only, it moved back across the wall to the mucosal side at a rate similar to that of sorbose, xylose, and other slowly absorbed sugars and much faster than that of glucose and galactose (Table V).

Anaerobically, the conversion of fructose to glucose was completely inhibited, while the movement of free fructose from the mucosal to serosal side was increased about 3-fold (Table II). In these experiments the

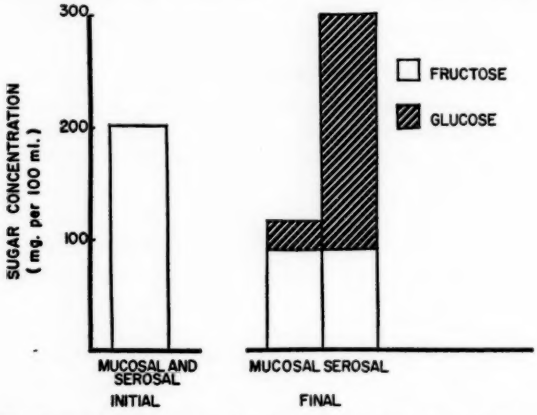

FIG. 1. The absorption of fructose by the hamster intestine. Bicarbonate-saline containing 200 mg. per cent fructose was placed on each side of the intestine (40.6 mg. dry weight), 5.0 ml. on the mucosal side and 1.4 ml. on the serosal side. Gas phase, 5 per cent CO_2 and 95 per cent O_2. Incubation for 1 hour at 37°.

amount of glucose found on the mucosal side was so small that it could not be measured with accuracy. The Q (fructose to glucose), therefore, refers only to the appearance of glucose on the serosal side. The increased movement of fructose anaerobically as shown in Table II is similar to that of sorbose and ribose (Table VI) and opposite to that of glucose and galactose.

When the fructose concentration on the mucosal side was 300 mg. per cent, an average of 75 per cent of the sugar appearing on the serosal side was glucose, with a concentration of 500 mg. per cent, the serosal concentrations of the two were approximately equal, and, with 1000 mg. per cent, the fructose appearing on the serosal side exceeded the glucose by about 5-fold. Under these conditions the conversion of fructose to glucose remained of the same order of magnitude (Q values between 1.7 to 2.3), while

the movement of the free fructose was roughly proportional to the concentration on the mucosal side.

Galactose—When the same concentration of galactose was placed on both sides of a sac of hamster intestine, it was transported across the wall against a concentration gradient (Table III). Previous studies of galactose absorption *in vitro* (22) were performed with glucose on the serosal side of the intestine, presumably to simulate physiological conditions. In the present study rapid absorption of galactose occurred in the absence of added glucose to either side. The gradients developed and total amounts transported are similar to those of glucose. The utilization of galactose

TABLE II

Effect of Anaerobic Conditions on Conversion of Fructose to Glucose and Movement of Fructose across Wall of Intestine

Everted sacs of intestine filled with about 0.6 ml. of sugar-free bicarbonate-saline. Sacs then placed in 25 ml. Warburg flasks with 3.0 ml. of bicarbonate-saline containing 200 mg. per cent fructose. The movement of sugar is from the mucosal to the serosal side. Gas phase, 5 per cent CO_2 and 95 per cent O_2. Incubation for 1 hour at 37°. The Q units represent microliters per mg. dry weight per hour.

Location	Q (fructose movement)		Q (fructose to glucose)	
	O_2	N_2	O_2	N_2
Jejunum	0.78	1.4	1.5	0
Ileum	0.57	1.7	1.3	0
Jejunum	0.32	3.1	3.1	0
Ileum	0.73	4.5	1.7	0
Jejunum	0.76	3.8	2.5	0
Ileum	1.1	3.8	2.3	0

by hamster intestine is similar to that of glucose. When 540 mg. per cent galactose was added to the mucosal side, the Q (galactose utilized) was 9.4 (nine experiments), while the corresponding value for glucose was 9.3 (seven experiments). Table III shows that the transport of galactose against a gradient was completely inhibited anaerobically. The possibility of the conversion of galactose to glucose was investigated by paper chromatographic methods. At the end of a few galactose experiments both serosal and mucosal solutions were run with *n*-butanol-pyridine-water, a solvent capable of separating glucose and galactose. No evidence for this conversion was found.

3-Methylglucose—This methylated glucose, the only synthetic sugar tested, was found to be absorbed against a concentration gradient by the hamster small intestine (Table IV). The rate of transport was about one-half that of glucose and galactose under similar conditions. No evidence

was found that 3-methylglucose was either utilized or converted to another sugar by the intestine.

Other Sugars—A sharp dividing line between two groups of sugars was

TABLE III

Transport of Galactose across Wall of Hamster Intestine

Sacs of intestine incubated in Warburg flasks with bicarbonate-saline for 1 hour at 37°. Gas phase, 5 per cent CO_2 and 95 per cent O_2 or 95 per cent N_2.

Location	Gas phase	Initial mucosal side	Initial serosal side	Final mucosal side	Final serosal side	Q (galactose transport)
		mg. per 100 ml.	*mg. per 100 ml.*	*mg. per 100 ml.*	*mg. per 100 ml.*	*µl. per mg. dry weight per hr.*
Jejunum	O_2	530	0	266	866	25
"	"	535	0	400	416	16
"	"	200	200	98	627	16
"	"	200	200	88	648	21
"	"	104	104	29	457	9
Ileum	"	104	104	25	405	13
Jejunum	N_2	104	104	112	137	0
Ileum	"	104	104	111	113	0

TABLE IV

Transport of 3-Methylglucose across Wall of Hamster Intestine

Sacs of intestine containing about 0.6 ml. of bicarbonate-saline were incubated in Warburg flasks containing 3.0 ml. of bicarbonate-saline for 1 hour at 37°. Gas phase, 5 per cent CO_2 and 95 per cent O_2.

Location	Initial mucosal and serosal side	Final mucosal side	Final serosal side	Q (sugar transport)
	mg. per 100 ml.	*mg. per 100 ml.*	*mg. per 100 ml.*	*µl. per mg. dry weight per hr.*
Jejunum	300	260	537	7.4
Ileum	300	262	482	5.4
Jejunum	150	133	326	8.6
Ileum	150	124	307	6.4

found in the ability of the hamster intestine to absorb them against a concentration gradient. Each sugar individually was added in the same concentration to both sides of the intestine and the ability of the intestine to transport them against a gradient was tested. Of the sugars tested glucose, galactose, and 3-methylglucose were transported against a gradient, while fructose, mannose, sorbose, xylose, arabinose, and ribose were not. The presence of glucose did not facilitate the absorption of the other sugars. This is illustrated by Fig. 2 which shows what occurred when both glucose

and xylose were added to each side of an intestinal sac. Glucose was absorbed against a final gradient of about 7-fold, while xylose concentrations remained practically unchanged. The utilization of the sugars other than glucose and galactose is small and further study is necessary to determine its significance.

Table V summarizes experiments in which sugar (0.03 M) was added to one side only and the rate of passage to the other was measured. Four sacs were prepared from each animal, two with a rapidly absorbed sugar

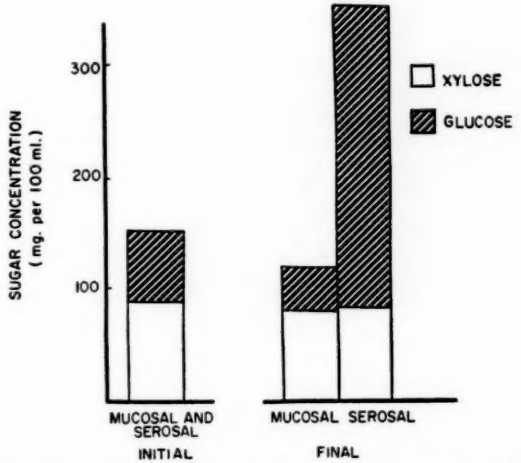

FIG. 2. The absorption of glucose and xylose by the hamster intestine. Bicarbonate-saline containing 64 mg. per cent glucose and 89 mg. per cent xylose was placed on each side of the intestinal sac (26 mg. dry weight), 10 ml. on the mucosal side and 1.4 ml. on the serosal side. Gas phase, 5 per cent CO_2 and 95 per cent O_2. Incubation for 1 hour at 37°.

and two with another sugar. This was done so that comparison of different sugars could be made with consecutive segments of the intestine in the same animal. The rate of movement across upper segments is faster than across lower ones. In the experiments in which glucose and galactose moved from mucosal to serosal sides the rate of passage was so rapid that the final concentration on the serosal side was often greater than that on the mucosal side, although at the end of many experiments the sugar was still moving down a concentration gradient, *i.e.* moving from a more concentrated to a less concentrated solution. All of the other sugars consistently showed lower concentrations on the serosal side than the opposite side at the end of an hour. The movement of either glucose or galactose

is very much faster than any other sugar tested, 5 to 10 times that of most sugars, and 30 times that of lactose. As has been mentioned before, fructose under these conditions produces glucose which appears almost exclusively on the serosal side. In these experiments the average Q (fructose to glucose) was 2.1.

When the direction of sugar movement is from the serosal to the mucosal side, the rate of movement of glucose is reduced 28-fold, while that of galactose is reduced 9-fold. The final concentration of glucose on the serosal side was between 300 to 400 mg. per cent and that on the mucosal side

TABLE V

Rate of Movement of Various Sugars across Wall of Hamster Intestine

Sacs of intestine were incubated in Warburg flasks for 1 hour at 37°. In the first series 0.03 M sugar solution was placed on the mucosal side only and the rate of appearance on the serosal side was measured. In the second series 0.03 M sugar solution was placed on the serosal side only. The numbers in parentheses represent the number of experiments performed. The Q units equal microliters per mg. dry weight per hour.

Sugar	Q (sugar movement)	
	From mucosal to serosal side	From serosal to mucosal side
Glucose	17 (6)	0.6 (8)
Galactose	15 (9)	1.6 (10)
Lactose	0.5 (3)	
Sorbose	1.7 (3)	4.8 (4)
Mannose	2.0 (6)	3.8 (3)
Fructose	2.7 (5)	3.0 (3)
Ribose	2.5 (5)	6.1 (3)
Xylose	4.7 (7)	3.9 (5)
Arabinose	1.8 (4)	3.8 (5)

was 3 to 4 mg. per cent (a value on the border line of significance). The final concentration gradient for galactose under similar circumstances was found to be 50-fold.

The effect of anaerobic conditions on the movement of four sugars is shown in Table VI. Four sacs were prepared from each animal, two absorbing glucose or galactose and two absorbing sorbose or ribose. Aerobic and anaerobic sacs for a given sugar were prepared from consecutive segments of intestine. With the two rapidly absorbed sugars anaerobic conditions reduced the rate of movement to one-third the aerobic rate. On the contrary, the slowly absorbed sugars, sorbose and ribose, show a 3-fold increase in rate of movement across the intestine under anaerobic conditions. Cooling the intestine from 37–0° greatly reduced the movement of

either glucose or xylose, in agreement with previous observations of Auchinachie, Macleod, and Magee (23).

Swelling of the intestinal tissue was regularly observed in the absence of oxygen (Table VII) and appeared to be independent of the sugar present.

TABLE VI

Effect of Anaerobic Conditions on Movement of Sugars across Intestine

Sugar solutions (0.03 M) were placed on the mucosal side of sacs of intestine and the rate of appearance on the serosal side was measured. Flasks gassed with 5 per cent CO_2 and 95 per cent O_2 or 95 per cent N_2. Results expressed as microliters per mg. dry weight per hour.

Sugar	Q (sugar movement)	
	O_2	N_2
Glucose	25.5	10.0
	15.3	4.0
	17.9	6.1
Average	19.5	6.7
Galactose	10.2	4.4
	18.5	9.4
	25.1	4.7
Average	17.9	6.1
Sorbose	0.47	4.25
	2.8	6.8
	1.7	7.7
Average	1.7	6.3
Ribose	1.8	6.8
	0.9	8.5
	2.5	5.9
Average	1.7	7.1

Swelling was also observed when the tissue was cooled to 0°, an effect previously reported (24).

Disaccharides and Starch—When the three disaccharides, sucrose, maltose, and lactose, were added to the mucosal side of the intestine or to an intestinal extract, they were hydrolyzed at different rates (Fig. 3). The amounts of sugar hydrolyzed by the enzymes maltase and sucrase were similar to each other and more than 10 times that of lactase, in agreement

TABLE VII

Water Content of Intestine Aerobically and Anaerobically

Following 1 hour's incubation, wet weight and dry weight were determined on the tissue. The numbers in parentheses represent the number of experiments performed.

Sugar	Water content, per cent water	
	O_2	N_2
Glucose	80.5 (3)	85.2 (2)
Galactose	81.7 (3)	86.3 (3)
Sorbose	80.1 (3)	85.4 (3)
Ribose	79.7 (2)	85.4 (2)

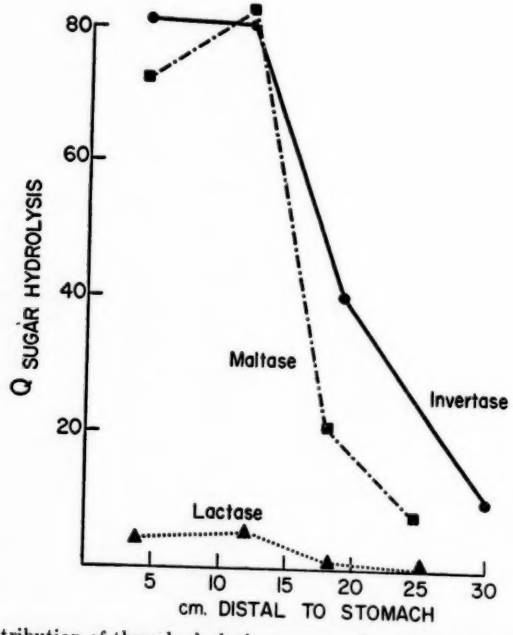

FIG. 3. Distribution of three hydrolytic enzymes along the intestine of the hamster. Carefully washed intestine was divided into four parts; the mucosa was scraped from the muscle and ground in a Potter-Elvehjem homogenizer. Portions of the whole homogenate were incubated at 27° in the presence of 2 per cent disaccharide solution and 0.02 M phosphate buffer (pH 6.8) for 30 or 60 minutes. Following deproteinization with $Ba(OH)_2$ and $ZnSO_4$, monosaccharides were estimated as reducing sugar directly on the filtrate (in the invertase experiment) or by chromatographic separation from the disaccharides and quantitative estimation after elution from paper chromatograms (in the other experiments). The units used are microliters per mg. dry weight per hour.

with earlier findings (25). All three activities were determined in the same extract of intestinal mucosa. Enzyme activity was about the same in the duodenum and upper jejunum but fell considerably in the lower jejunum and ileum. It is interesting to note that the rate of hydrolysis of maltose and sucrose is at least 2 to 3 times the maximal rate at which the glucose residue can be absorbed.

An experiment illustrating sucrose absorption by the hamster intestine is shown in Fig. 4. Although sucrose was split into both glucose and fruc-

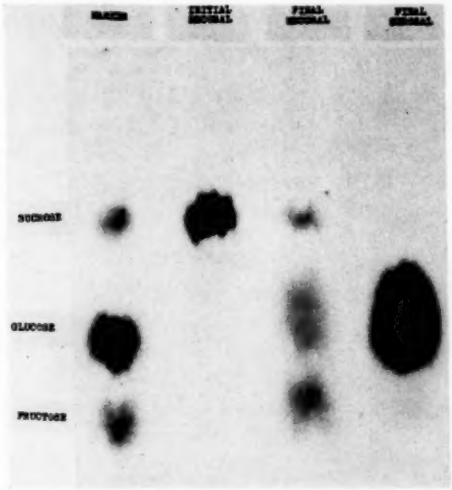

FIG. 4. The absorption of sucrose by a sac of hamster intestine. An everted sac of jejunum (103 mg. dry weight) containing 2.5 ml. of sugar-free bicarbonate-saline was placed in a 125 ml. Warburg flask with 5.0 ml. of bicarbonate-saline containing 1 per cent sucrose. 30 μl. of each solution were placed on a paper chromatogram and run descending for 64 hours with *n*-butanol-acetic acid-water as the solvent.

tose on the mucosal side, only glucose appeared on the opposite side in appreciable amounts. Quantitative estimation of each sugar was performed by elution of sugars from strips of chromatographic paper and estimation of ketose and reducing sugar. In this experiment most of the sucrose was hydrolyzed during the 1 hour incubation, leaving only 90 mg. per cent on the mucosal side and 6 mg. per cent on the serosal side. Glucose was the predominant sugar on the serosal side (490 mg. per cent), although a small amount of fructose (62 mg. per cent) was also present. The fructose concentration on the mucosal side (268 mg. per cent) was more than double that of the glucose (103 mg. per cent). Considerable utilization of fructose by the tissue or its conversion to glucose must

have occurred, since relatively little fructose was found at the end of the experiment.

When larger amounts of sucrose (5 per cent solutions) were placed on the mucosal side, their rate of hydrolysis to glucose and fructose exceeded

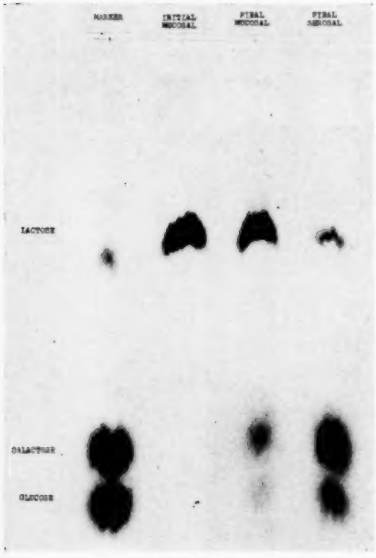

FIG. 5

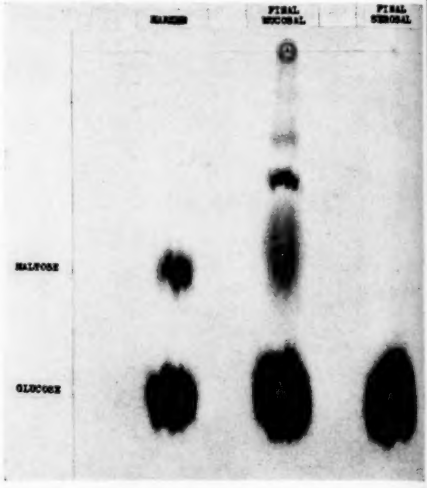

FIG. 6

FIG. 5. The absorption of lactose by a sac of hamster intestine. An everted sac of jejunum (87 mg. dry weight) containing 2.2 ml. of sugar-free bicarbonate-saline was placed in a 125 ml. Warburg flask with 5.0 ml. of bicarbonate-saline containing 1 per cent lactose. 30 μl. of each solution were placed on a paper chromatogram and run descending for 40 hours with *n*-butanol-pyridine-water as the solvent.

FIG. 6. The absorption of starch by a sac of hamster intestine. A sac of jejunum (80 mg. dry weight) containing 2.0 ml. of sugar-free bicarbonate-saline was placed in a 125 ml. Warburg flask with 5.0 ml. of bicarbonate-saline containing 1 per cent soluble starch. 100 μl. of final solutions were placed on a paper chromatogram and run descending for 20 hours with *n*-butanol-pyridine-water as the solvent.

their rate of absorption and they accumulated on the mucosal side. In these experiments an additional slow running spot appeared (containing fructose) on the paper chromatogram (*n*-butanol-acetic acid-water) from the solution on the mucosal side. This may be similar to one of the oligosaccharides described by Bacon and Edelman (26) and Blanchard and Albon (27) which appear during the hydrolysis of sucrose by yeast invertase. A similar slow running spot was seen during the hydrolysis of 1 per

cent maltose (an effect reported with intestinal extracts by Bacon, Bacon, Clarke, and Smyth (28)).

When 1 per cent lactose was added to the mucosal side of the intestine, only a small amount of hydrolysis occurred (Fig. 5). Quantitative chromatograms in this experiment showed that the concentrations of the two monosaccharides were similar on the mucosal side (20 mg. per cent), while on the serosal side the concentration of galactose (73 mg. per cent) exceeded that of glucose (55 mg. per cent). Apparently the tissue utilizes glucose in preference to galactose.

A 1 per cent solution of soluble starch was placed on the mucosal side of a sac of hamster intestine for 1 hour. A chromatogram of the final solutions on both sides is shown in Fig. 6. When the initial starch solution was run in this solvent, it remained on the starting line, as indicated by iodine spray, but contained no material which reacted with the benzidine spray. At the end of 1 hour the mucosal solution did not react with iodine but gave a number of "slow" running spots on the chromatograms. The only sugar found on the serosal side was glucose.

DISCUSSION

Two important criteria of active transport of uncharged molecules are (1) movement of the substance against a concentration gradient, and (2) inhibition of transport as a result of inhibition of the energy-yielding reactions. With methods *in vivo* it is difficult or impossible with most sugars to test the first criterion, although in the case of glucose it was successfully accomplished by Bárány and Sperber (29). In 1949 Fisher and Parsons developed the first well oxygenated preparation of rat intestine *in vitro* and showed the transport of glucose and galactose against a concentration gradient (30, 22). In the present study glucose, galactose, and 3-methylglucose but not mannose, sorbose, xylose, arabinose, or ribose are found to be transported against a concentration gradient by hamster small intestine. Fructose was found to be unique. Although the glucose which is formed from fructose is transported against a concentration gradient to the serosal side, the fructose that reaches the serosal side appears to move only by passive diffusion. Although the conversion of fructose to glucose both *in vivo* (31, 13) and *in vitro* (32, 21) has previously been well established, the passive nature of the fructose movement across the intestine has not been adequately emphasized.

Evidence for the second criterion of active transport, inhibition of transport with metabolic inhibitors, has been obtained by a number of older experiments *in vivo*. Iodoacetate has been shown to inhibit strongly the absorption of glucose and galactose but not fructose, mannose, xylose, and arabinose (4). Experiments with phlorizin are of special interest, since

the inhibition produced by this poison appears to be more specific than that produced by iodoacetate. It inhibits the intestinal absorption of glucose, galactose, and fructose, having no effect on the absorption of xylose, arabinose, sorbose, amino acids, fat, or salts (5, 6, 33).

Csáky (34) first showed that 3-methylglucose is absorbed by rat intestine at a similar rate to that of glucose. The hamster intestine, although it transports this sugar, is unable to utilize it in the same manner that it does other transported sugars. Apparently endogenous substrate is adequate to maintain the transporting mechanism, since no added substrate is needed for 3-methylglucose transport.

Experiments *in vitro* have shown a sharp separation between two groups of sugars by the effect of anaerobic conditions on the sugar movement across the intestine. The absorption of either glucose or galactose against a concentration gradient is completely inhibited by anaerobic conditions, while none of the other sugars tested are absorbed against a gradient in the presence or absence of oxygen. When sugars are moving down a concentration gradient (from a higher to a lower concentration), anaerobic conditions are found to inhibit the movement of glucose and galactose, while the same conditions stimulate the movement of sorbose, ribose, and fructose (Table VI). Although there is somewhat more tissue disintegration anaerobically than aerobically, this is not considered the major factor in the increased rate of movement of sorbose, ribose, and fructose. Increased diffusion of sugars across the rabbit or cat intestine *in vitro* was observed by Auchinachie, Macleod, and Magee (23) after treatment with NaF, KCN, heat, or prolonged exposure to air. The significance of these effects is not, as yet, fully understood.

The first criterion of active transport, movement against a concentration gradient, sometimes cannot be met experimentally, as was the case of sugar absorption by the intestine in living animals. This, however, does not necessarily exclude active transport as a possible mechanism. It can be shown with methods *in vitro* that glucose and galactose, when moving across the intestine down a concentration gradient, depend in a large part upon an active transport mechanism requiring expenditure of energy; relatively smaller amounts move across by simple diffusion. With 250 mg. per cent glucose on the mucosal side and no sugar initially present on the serosal side, the movement of glucose is inhibited 90 per cent by NaCN, quinine, dinitrophenol, and anaerobic conditions (21); with 500 mg. per cent on the mucosal side, the inhibition by anaerobic conditions was about 65 per cent. Further evidence of the relatively small rôle of diffusion in the absorption of glucose (at least at concentrations of 500 mg. per cent or less) is given by the fact that the rate of transport either up or down a concentration gradient is of the same order of magnitude. The transport values reported

in Table V for glucose movement down a gradient are similar to those previously reported (14) for movement up a gradient. Similar effects were found by Fisher and Parsons (22) for galactose. It appears that a substance which can be transported up a concentration gradient is also given an active impetus when passing down a concentration gradient by the active transporting mechanism.

There is evidence that some sugars which are not absorbed against a concentration gradient are phosphorylated. For example, relatively large amounts of fructose-1-phosphate accumulate when fructose is being absorbed from the lumen of the intestine. The evidence presented here, however, suggests that fructose-1-phosphate is not immediately dephosphorylated in such a manner as to give active transport of fructose. Instead, a more complicated series of reactions occurs with glucose as the end-product. Mannose, xylose, and ribose are also phosphorylated (10, 12), although they cannot be absorbed against a concentration gradient (at least by hamster intestine). Although the explanation for this phenomenon is not entirely clear, it is certain that the mere phosphorylation of a sugar by the intestine does not prove that it is transported by an active process.

SUMMARY

1. When fructose is placed on the mucosal side of hamster intestine *in vitro*, some of the sugar is converted to glucose which appears predominantly on the serosal side. This conversion is completely inhibited anaerobically, while the movement of free fructose across the wall increases. Evidence is presented which suggests that the movement of free fructose is by passive diffusion.

2. Galactose is absorbed rapidly against a concentration gradient in this preparation and this absorption is completely inhibited anaerobically.

3. Glucose, galactose, and 3-methylglucose were the only sugars which were absorbed against a concentration gradient among those tested (fructose, sorbose, mannose, arabinose, xylose, and ribose).

4. Sucrose, maltose, lactose, and starch were added to the mucosal side of the intestine and the rate of hydrolysis, as well as the rate of absorption of the split products, was studied.

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STUDIES ON THE ALKALINE HYDROLYSIS OF LECITHIN: SYNTHESIS OF CYCLIC 1,2-GLYCEROPHOSPHATE*

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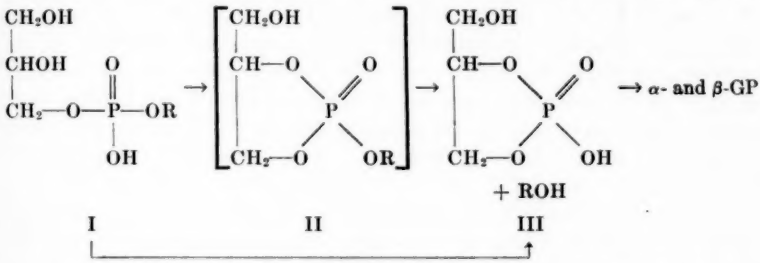
Markham and Smith (1) have isolated and identified cyclic 2′,3′-nucleotides as intermediates in the hydrolysis of ribonucleic acid and have shown that formation of these cyclic phosphate esters is responsible for the migration of the phosphate group from the 3′- to the 2′-hydroxyl. Other cyclic phosphate esters have also been described (pantetheine-2′,4′-phosphate (2), cyclic glucose phosphate (3), and riboflavin-4′,5′-phosphate (4)).

In the hydrolysis of derivatives of glycerol phosphate a similar migration of the phosphate group, from the α to the β position, occurs. Thus α-methyl GPA[1] (5), L-α-GPC (6), L-α-GPE (7), and synthetic L-α-lecithin (8) all yield a mixture of α- and β-GP on alkaline as well as on acid hydrolysis. Verkade *et al.* (9) postulated a cyclic intermediate to explain the acid-catalyzed migration of the phosphate group in GPA, and Chargaff's demonstration (10) of the intramolecular nature of the reaction supports this view. Baer and Kates (8) have postulated the formation of a cyclic phosphate *triester* (II) to explain the results of kinetic studies of the al-



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[1] The following abbreviations are used: GPA, glycerophosphoric acid; GP, glycerophosphate (preceded by alkyl or cation substituent where necessary); CGP, cyclic 1,2-glycerophosphate; GPG, glycerophosphatogen; GPC, glycerylphosphorylcholine; DPL, dipalmitoyllecithin; GPE, glycerylphosphorylethanolamine.

kaline hydrolysis of synthetic lecithins, but the alternative possibility is not excluded that a concerted loss of choline occurs, forming a cyclic glycerol phosphate (CGP) (III) in a single reaction.

Despite these numerous references to the cyclic glycerol phosphate, this interesting intermediate has not been isolated or directly detected. However, it is interesting to note that Fleury (11) obtained a new glycerol- and phosphate-containing compound (glycerophosphatogen (GPG)) by hydrolysis of lecithin in 0.5 N aqueous methanolic potassium hydroxide at 37° for 65 hours. This material had little titratable acidity over the range pH 4 to 9 (the pK_a values for α-GPA are 1.40 and 6.44 (12)), gave a faint or no precipitate with lead acetate, but yielded GP, whose lead salt is water-insoluble, on more vigorous hydrolysis. Using similar conditions to hydrolyze synthetic L-α-DPL, Baer and Kates (8) obtained a fraction which contained 94 per cent of the phosphorus, but from which lead GP could not be obtained.

Fleury's work was of interest since the properties of GPG were not inconsistent with those of a cyclic glycerol phosphate contaminated with GP. The presence of a methyl ester of GPA was also possible, although Fleury reported that no methanol was obtained on hydrolysis of GPG.[2]

This paper deals with an investigation of the hydrolysis products obtained from lecithin under the above conditions, and with the synthesis and properties of CGP.

Fleury's procedure was repeated with the exception that the fatty acids were removed from the methanol hydrolysate under milder conditions (petroleum ether extraction at pH 2.5 to 3.0 as compared to concentration of a solution at pH 3 to dryness and extraction with benzene). The phosphate-containing hydrolysis products were separated into two fractions by chromatography on Dowex 2. The slower moving fraction contained 30 per cent of the phosphorus and was identified as GP by paper chromatography. The major fraction gave no precipitate with lead ion, contained no titratable acidity over the range pH 4 to 9, but yielded GP on further hydrolysis. It was crystallized in fine needles as the barium salt from ethanol-water; analysis showed it to be the methyl ester of GPA. On paper chromatography in isopropanol-5 N ammonia (2:1), it gave the same R_P (0.60) as the major reaction product of GPA with dimethyl sulfate or diazomethane.

With the synthesis of CGP (described below), it became possible to examine the lecithin hydrolysates directly by paper chromatographic methods.[3] Lecithin was hydrolyzed in isopropanol, ethanol, and water under

[2] Since this work was completed, Fleury and Le Dizet (13) have reported that methyl GP is the major component of GPG.

[3] CGP (R_P = 0.59) and methyl GP were indistinguishable in isopropanol-am-

the conditions of Fleury and Le Dizet (14) and in aqueous barium carbonate slurry as described by Markham and Smith. In no case was a spot found corresponding to the cyclic compound. Ethanol hydrolysates gave a spot with R_f 0.70 that corresponded to the ethyl ester of glycerophosphoric acid obtained by acid decomposition of CGP in ethanol, but no esterification was detected in the isopropanol hydrolysate.

In further hydrolysis studies, lecithin was replaced with the simpler diesters, barium benzyl GP and L-α-GPC.[4] Paper chromatography of barium benzyl GP, refluxed with barium carbonate slurry 1 hour, and L-α-GPC, hydrolyzed 6 hours at 80° and pH 11 in carbonate buffer, revealed faint spots corresponding to CGP in addition to starting material and GP; however, the amount was too small for isolation or additional characterization.

The cyclic 1,2-glycerophosphate was prepared from β-GPA with trifluoroacetic anhydride in a modification of the procedure reported by Brown, Magrath, and Todd (15) for the preparation of cyclic 2′,3′-nucleotides. The trifluoroacetyl derivatives of GP and CGP were decomposed with ammonia in anhydrous isopropanol. Ammoniacal methyl and benzyl alcohol were unsatisfactory solvents, yielding large amounts of the corresponding esters of GP as by-products. Ammonium GP precipitated completely during the reaction and, on evaporation of the solvent from the supernatant fluid, ammonium CGP was obtained, contaminated with ammonium trifluoroacetate and trifluoroacetamide. The latter was removed by sublimation. The ammonium salts were converted to barium salts[5] with IRC-50-Ba^{++}, and the lyophilized eluent extracted with acetone to remove barium trifluoroacetate. A small amount of contaminating isopropyl GP was separated by chromatography on Solka-Floc with methanol-isopropanol (5:1), and the pure barium CGP was crystallized from aqueous ethanol.

The electrometric titration of pure barium CGP showed no secondary phosphate dissociation. The infra-red spectra of this salt showed the presence of a primary alcoholic group (O—H stretching at 3330 $cm.^{-1}$ and strong C—O stretching at 1072 $cm.^{-1}$) and no indication of a secondary alcoholic group, thus confirming the 1,2 structure.

Pure barium CGP is stable in water over the range pH 3.5 to 8.0 for at least 72 hours at room temperature. At pH 1.5 and 13, partial decomposition into glycerophosphate occurs in 3 hours at the same temperature, and the conversion is complete in 0.1 N hydrochloric acid or 0.5 N sodium hydroxide solution after 3 hours at room temperature. On standing at room

monia; however, the isopropyl and ethyl esters of GP had sufficiently higher R_f values to allow the use of these alcohols in hydrolysis studies.

[4] Kindly supplied by Dr. D. J. Hanahan, University of Washington.

[5] Attempts to prepare the barium salt by way of the free acid were unsuccessful. After passage over IR-120-H^+, the CGP was largely converted to GP. The presence of trifluoroacetate seemed to catalyze the reaction.

temperature in absolute ethyl or benzyl alcohol acidified with trifluoroacetic acid, CGP is converted into the corresponding ester of GPA. The benzyl ester was prepared as the barium salt and characterized by its ultraviolet spectrum.

These data establish that the pure cyclic glycerophosphate is comparable in stability to the nucleotide analogues; however, it seems unlikely that the cyclic ester can be actually isolated as an intermediate in the alkaline hydrolysis of lecithin.

EXPERIMENTAL

Lecithin was prepared from egg yolk (16, 17) and hydrolyzed under the conditions described by Fleury (11). In a typical experiment 8.5 ml. of 30 per cent aqueous potassium hydroxide were added to a solution of 8.3 gm. of lecithin dissolved in 85 ml. of methanol. The solution was kept at 37° for 65 hours, then adjusted to pH 2.5 with 5 N sulfuric acid. After standing overnight at −10°, the precipitate was removed by centrifugation and washed twice with cold methanol. The combined solutions were extracted three times with 150 ml. of hexane, and the hydrolysate was adjusted to pH 6 to 7 with 2 N sodium hydroxide solution. The methanol was removed *in vacuo* and the residue made up in water for use in ion exchange chromatography.

In one experiment 30 per cent potassium hydroxide in anhydrous methanol instead of water was added to reduce the yield of GP. Hydrolyses were also run with ethanol, isopropanol, and water as solvents. 1 ml. aliquots were removed from the above hydrolyses at intervals of 2, 6, 24, and 48 hours and prepared for paper chromatography.[6] Sulfuric acid (0.8 N) was added to give a pH of 3, the precipitate was centrifuged off, and the solution extracted three times with hexane. The pH was adjusted to 6 with potassium hydroxide and the solution concentrated to 0.2 ml. under reduced pressure. This method does not completely remove fatty acids; however, their presence did not interfere with ion exchange procedures, although it caused further elongation of the GP spot in paper chromatography.

Separation of Methyl Glycerophosphate—1 ml. of aqueous methanolic hydrolysate (pH 3.0; 3.4 mg. of P) was added to Dowex 2-OH^- (200 to 400 mesh, 9 ml. of resin, 0.65 × 28.5 cm. column). The column was washed

[6] 10 to 40 γ of phosphorus were applied to Schleicher and Schüll blue ribbon paper, which was equilibrated 4 hours with the solvent system (isopropanol-5 N ammonia (2:1), unless otherwise noted) and run for 24 hours. Phosphate was detected by the method of Bandurski and Axelrod (18). R_F values varied greatly with phosphorus concentration, temperature, and age of solvent, so that controls were always run simultaneously. Disodium α- and β-GP consistently gave an elongated spot of R_F 0.25 to 0.35.

with 80 ml. of distilled water and then eluted with 0.08 N sodium hydroxide at a flow rate of 4 to 5 ml. per hour. 9 ml. fractions were collected and analyzed for phosphorus by the method of Fiske and Subbarow. Sharp peaks occurred at 90 and 220 ml. of eluate. The faster moving fraction (I) contained 2.31 mg. of P, and the slower (II), 1.03 mg. of P. The material in the peak tubes was neutralized with IRC-50-H^+ and concentrated to 0.2 ml. Paper chromatography showed Peak II identical with GP, while Peak I gave a small spot corresponding to GP and a major spot with R_F 0.60. To determine whether the appearance of GP in Peak I could be due to slow hydrolysis of the methyl ester in dilute base, 50 mg. of purified barium methyl GP were dissolved in 10 ml. of 0.1 N sodium hydroxide and the solution was allowed to stand at room temperature. After 2 days, GP was detected in the mixture and appeared as the major spot after 5 days.

Methyl GP was purified and isolated as the barium salt. 100 ml. of absolute methanolic hydrolysate (4.0 mg. of P per ml.; pH 3.5) were added to Dowex 2-OH^- (100 ml. of resin, 1.5 × 55 cm.) and, after washing with 120 ml. of water, the column was eluted with 0.05 N potassium hydroxide at a flow rate of 17 ml. per hour. The 400 to 900 ml. fraction of eluate was neutralized over IRC-50-H^+ and lyophilized to a white syrup. This material was dissolved in 100 ml. of water, passed through IR-120-H^+, and then immediately through IRC-50-Ba^{++}, and the eluate lyophilized to give a glassy white solid which contained a small amount of GP in addition to methyl GP. 1 gm. of this material was treated with 1 ml. of water and the undissolved solid was centrifuged. To the supernatant solution were added 1 ml. of water and 25 ml. of ethanol, and the solution was warmed. The flocculent white precipitate (0.77 gm.) which formed on cooling was centrifuged, washed twice with cold absolute ethanol, and once with acetone. 0.2 gm. of this material was dissolved in 1 ml. of water and 24 ml. of hot absolute ethanol were added. On cooling slowly to room temperature, 70 mg. of rod-like crystals formed on the beaker. On standing 2 days at room temperature, an additional 50 mg. of long slender needles formed in the solution. Samples were dried to constant weight at 140° and 0.5 mm. pressure.

$BaC_6H_{20}P_2O_{12}$.	Calculated.	C 18.93, H 3.97, P 12.2
(507.65)	Found.	" 19.00, " 4.00, " 12.3

Titration of 18.2 mg. of barium methyl GP in 3 ml. of water (after adding 0.02 ml. of 0.1 N sodium hydroxide) with 0.1 N hydrochloric acid revealed no titratable acidity over the range pH 4 to 10.

Preparation of Cyclic 1,2-Glycerophosphate—A 5 per cent aqueous solution of commercial sodium β-GP was passed through a column of IR-120-H^+ and the effluent was lyophilized. The viscous β-GPA was dried

over phosphorus pentoxide *in vacuo* to constant weight. This acid (1.636 gm.) was mixed with 40 ml. of trifluoroacetic anhydride and the mixture was kept at room temperature overnight. The solvent was removed *in vacuo* at room temperature and the syrupy residue was dried for 12 hours *in vacuo* over sodium hydroxide to remove remaining trifluoroacetic acid. The viscous residue was dissolved in 50 ml. of anhydrous isopropanol and the solution added slowly with stirring at 4° to 50 ml. of anhydrous isopropanol saturated with ammonia. After stirring for 1.5 hours, the white precipitate (0.64 gm.) was removed by centrifugation and identified as ammonium GP by paper chromatography. The solvent was evaporated *in vacuo* at room temperature, and the residue was kept at 0.1 mm. at room temperature until no more white sublimate (trifluoroacetamide) was found in the trap.

The crude ammonium CGP thus obtained (yield 2.17 gm.) gave no precipitate with 4 per cent aqueous lead acetate. The crude salt was tested on paper chromatography and gave a major spot with R_F 0.6 and an additional faint spot with R_F 0.72.

The crude ammonium CGP thus obtained was dissolved in 400 ml. of ice-cold water and passed slowly (flow rate 1 ml. per minute) through a column of IRC-50-Ba^{++}, and the column was washed with 100 ml. of water. The effluent (pH 4.5) and the wash water were combined and lyophilized. The above operations were all performed at 4°. The colorless glassy residue was washed ten times with 8 ml. of dry acetone to give 1.00 gm. of white powder. Paper chromatography of this material revealed a small amount of impurity with R_F value 0.72 in addition to the CGP (R_F 0.6).[7]

Purification of Barium Cyclic Glycerophosphate—The crude barium CGP (0.56 gm.) was dissolved in 2 ml. of anhydrous methanol, and adsorbed on a column of Solka-Floc (1 × 49 cm.) which was prepared in methanol and isopropanol (5:1). The material was eluted with methanol-isopropanol (5:1) at a flow rate of 9 ml. per hour and 6 ml. fractions were collected. Phosphorus was detected qualitatively in Tubes 7 to 10 and 32 to 88. Upon removal of the solvent *in vacuo* at room temperature from the faster moving fractions, an oily residue with R_F 0.72 was obtained.

[7] The paper chromatogram of the barium CGP showed an additional tailing spot from the starting point when the ammonia-containing solvent system was used. This tailing spot could be caused by partial decomposition of the CGP into GPA during chromatography. Thus a similar tailing spot was observed for barium GP with the same solvent system. But, when a solvent system containing only isopropanol and water (1:1) was used for barium CGP, no additional spot other than the major one was observed. The latter solvent system is convenient to check crude barium CGP for contamination with barium GP, since the latter does not travel on the paper with this solvent system.

The slower moving fraction, when treated as above, yielded 0.277 gm. of white crystalline powder. It was washed with 10 ml. of methanol to remove resinous impurities, with pure acetone, and dried at room temperature (yield 0.136 gm.).

Recrystallization of Barium Cyclic Glycerophosphate—75 mg. of this solid were suspended in 0.75 ml. of absolute ethanol, 0.2 ml. of distilled water was added to the suspension to dissolve the crystals, and the solution was filtered. To the filtrate 0.5 ml. of absolute ethanol was added under cooling and stirring. From the cloudy solution microscopic crystals precipitated after about 10 minutes. The recrystallization was repeated once more, giving 29 mg. of pure salt. The sample for analysis was dried over phosphorus pentoxide at room temperature for 24 hours under 3 mm. pressure.

$(C_3H_6O_6P)_2Ba$.	Calculated.	C 16.25, H 2.75
(443.5)	Found.	" 16.22, " 2.97

Stability of Cyclic Glycerophosphate in Aqueous Solutions—6 mg. of barium CGP were dissolved in 0.2 ml. of buffer at pH 1.5, 3.5, 6.0, and 8.0, and in 0.1 N hydrochloric acid, 0.1 N and 0.5 N sodium hydroxide solution. The solutions were kept at room temperature and aliquots were removed at intervals for paper chromatography. Between the range pH 3.5 and 8.0 the compound was stable and no GP was detected in 72 hours. For both solutions of pH 1.5 and 0.1 N sodium hydroxide a rapid decomposition of the compound into GP was observed after 3 hours. The CGP spot disappeared entirely in both 0.5 N sodium hydroxide and 0.1 N hydrochloric acid after 3 hours and in buffer, pH 1.5, solution after 72 hours.

Decomposition of Barium Cyclic Glycerophosphate in Ethanol and in Benzyl Alcohol with Trifluoroacetic Acid—3 mg. of barium CGP were added to 1 ml. each of absolute ethanol and benzyl alcohol containing a drop of trifluoroacetic acid and kept for 3 days at room temperature. Every 24 hours aliquots were removed for paper chromatography. A gradual disappearance of the spot for CGP occurred with the appearance of new spots with R_F values of 0.70 and 0.77 for the ethanol and benzyl alcohol solutions, respectively. After 72 hours standing, complete disappearance of the CGP spot was observed for both solutions.

Isolation and Identification of Benzyl Glycerophosphate—The mixed anhydride of the GPA with trifluoroacetic anhydride obtained from 0.605 gm. of sodium β-GP was decomposed as above with anhydrous benzyl alcohol and ammonia instead of anhydrous isopropanol. The reaction mixture was shaken with water and the water layer washed with ether. The crude ammonium CGP obtained after evaporation of water at room temperature was fractionated with a Solka-Floc column (4.5 × 23 cm.)

and isopropanol-5 N aqueous ammonia for elution. Three fractions were obtained. The first contained the major product with R_F 0.77. After removal of the solvent at room temperature from this fraction, a syrupy ammonium salt (0.1 gm.) was obtained. A 0.3 per cent solution of this salt was passed through a column of IRC-50-Ba^{++} as above, and the effluent gave a white powder on lyophilization. It was precipitated twice from water solution with absolute ethanol, giving a white powder.

A 10^{-3} M water solution of this compound was used to obtain the ultraviolet spectrum. The compound showed a maximal absorption at 257 mμ (ϵ_{397}) and two inflections at 252 mμ (ϵ_{320}) and 262 mμ (ϵ_{342}), which are specific absorption bands for the phenyl radical (19).

SUMMARY

Cyclic 1,2-glycerophosphate has been synthesized and characterized as the crystalline barium salt.

Hydrolysis of lecithin in alkaline methanol yields as the main product the methyl ester of glycerophosphoric acid. No evidence was obtained for the presence of the cyclic phosphate ester in the hydrolysis products. The instability of the synthetic cyclic glycerophosphate in alkaline solutions makes it appear unlikely that the cyclic ester will be found in alkaline hydrolysates of lecithin.

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